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Characterization of inter-animal variation in the innate immune response of the bovine and its relation to S. aureus mastitis.

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CHARACTERIZATION OF INTER-ANIMAL VARIATION IN THE INNATE IMMUNE RESPONSE OF THE BOVINE AND ITS RELATION TO *S. AUREUS* MASTITIS

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Abstract

Mastitis represents one of the major economical and animal welfare concerns within the dairy industry. Animals affected with this disease can experience a range of clinical symptoms from mild discomfort and swelling of the udder to a severe systemic inflammatory response that could result in the death of the animal. This range of responses is due to differences in pathogen, environment, and inter-animal differences in their innate immune response. A dermal fibroblast model was used to predict the magnitude of an animal's innate immune response towards an intra-mammary *S. aureus* challenge. Animals whose fibroblasts exhibited a low response phenotype, characterized by lower levels of IL-8 following *in vitro* immune stimulation, suffered less mammary tissue damage and a less severe reduction in milk quality following the *in vivo S. aureus* challenge as compared to animals classified as high responders. Furthermore, the heightened inflammatory response of the high responders offered no advantage in bacterial clearance. For a *S. aureus* infection, the lower response phenotype is preferred.

To further explore inter-animal variation in the innate immune response, fibroblast cultures were established and challenged with LPS from two breeds of cattle, Holsteins, a dairy breed and Angus, a beef breed. Cultures from Holstein animals exhibited a higher responding phenotype than cultures from Angus animals. As these two breeds undergo selection for different traits and are reared differently as calves, whole transcriptome analysis (RNA-Seq) and DNA methylation analysis (Methylated CpG Island Recovery Assay; MIRA-Seq) of their fibroblasts was completed to examine the genetic and epigenetic basis for the contrasting responses. RNA-Seq revealed several immune associated genes that were expressed at higher levels in Holstein cultures compared to Angus cultures, including TLR4, IL-8, CCL5, and TNF-α, both basally and following LPS exposure. Although MIRA-Seq analysis revealed 49 regions with differential methylation between the Holstein and Angus cultures, overall, the methylation of the fibroblast genome was similar between these breeds. A combination of genetic and epigenetic factors seems to contribute to the breed-dependent differences observed between Holstein and Angus fibroblasts.

Early life exposure to bacterial compounds or inflammatory mediators can have long-term effects on the magnitude of an animal's innate immune response, and may contribute to inter-animal variation in this response. To determine if an early life exposure to LPS would modify the response to a subsequent LPS challenge in dairy animals, neonatal Holstein calves were treated with LPS or saline at 7 days of age and subsequently challenged with LPS 25 days later. Calves that received LPS at 7 days of age had greatly elevated levels of plasma IL-6 and TNF-α compared to calves that received saline, indicating a substantial inflammatory response. However, following the subsequent LPS challenge completed on all calves, there were no differences in plasma IL-6 and TNF-α between the LPS- and saline- treated calves. Alternative exposure strategies in calves may generate the long-term effects observed in other model systems.

There is a wide range in the responses observed in the innate immune response of the bovine. Animals with a lower innate immune response effectively clear the infection, but avoid the collateral tissue damage from excessive inflammation. Therefore, it seems that a reduced innate immune response would be more beneficial to the dairy cow.

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Table of Contents

List of Tables

List of Figures

Chapter 2: Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with *Staphylococcus aureus*

Chapter 3: Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs with major differences in the methylome

Chapter 4: Neonatal LPS exposure does not diminish the innate immune response to a subsequent LPS challenge in Holstein bull calves

Appendix A: LPS response of dermal fibroblasts isolated from genomically tested dairy cattle

Appendix B: A comparison of LPS-response of dermal fibroblasts isolated from young Angus and Holstein animals

Chapter 1: Literature Review

1.1 Bovine Mastitis and its Consequences

Mastitis, or inflammation of the mammary gland, is an economically important disease that affects approximately 20% of the cattle in dairy herds within the United States (USDA, 2007). A single case of clinical mastitis can cost the producer approximately $200, depending on many factors including the causative pathogen, the current milk price, the production level of the cow, and the cost of feed (Bar et al., 2008). Within the United States, mastitis has been calculated to cost the dairy industry $2 billion annually (G.M. Jones, 2009), and the reduction in milk yield from the affected animal is one of the largest contributors to this financial loss (Zhao and Lacasse, 2008). A comprehensive study of several New York state dairy farms revealed that many of the cattle that experience repeated cases of clinical mastitis during a single lactation are animals producing the largest volume of milk, resulting in even greater milk losses (Bar et al., 2007). Along with a loss in quantity, the recruitment of immune cells into the gland to clear the infection elevates the somatic cell count (SCC) of the milk, and if the SCC increases above 750,000 cells/ml, the milk is no longer marketable within the United States (Ma et al., 2000). Additional factors that contribute to the cost associated with mastitis are antimicrobial and supportive treatments administered to the animal and if warranted, the premature culling of the animal from the herd (Bar et al., 2008). If the inflammatory response is severe enough, the secretory tissue of the mammary gland may become damaged, thereby inhibiting the ability of the animal to return to its full productive potential (Akers and Nickerson, 2011). A greater profit loss can occur if the affected animal is culled and she was in her first lactation, as the investment into rearing that animal from a calf into a productive adult cow has not yet been recovered by the

producer. The cost of raising a single dairy calf from birth to freshening is one of the biggest expenditures on a farm, second to the feed costs of the milking herd. It has been estimated that the average producer will spend approximately $2,000 on a replacement animal, depending on several factors including current feed prices, management practices on the farm, and age at first calving (Heinrichs et al., 2013, Akins, 2015).

Mastitis is a disease that raises animal welfare concerns for both the dairy producer and the consumer. Affected animals can experience pain and discomfort from the infected gland, and in a recent study, Norwegian dairy producers ranked the pain associated with severe mastitis similar to a dystocia or distal limb fracture (Leslie and Petersson-Wolfe, 2012). Additionally, the perception of the consumer on how adequately the welfare of the animal is being addressed during an illness is important, as consumers want to be assured that the animals producing the milk they purchase are being treated humanely (Leslie and Petersson-Wolfe, 2012). Because mastitis affects the animal and the producer, as well as the public, a better understanding of the initiation, progression, and resolution of the inflammatory response generated towards a mammary infection would benefit the dairy industry at several levels.

1.2 Infectious Agents of Bovine Mastitis

Mastitis can be caused by a variety of pathogens, including viruses (Wellenberg et al., 2002), fungi (Williamson and di Menna, 2007), and bacteria (Oliveira et al., 2013). Among these pathogens, bacteria are the most common and are broadly grouped into contagious or environmental. Bacteria that are spread from cow-to-cow or between quarters of the same animal during milking are considered contagious and include

Staphylococcus aureus, *Streptococcus agalactiae*, and *Mycoplasma* species, among others (Oviedo-Boyso et al., 2007). Alternatively, infection by environmental pathogens, such as *Escherichia coli*, *Streptococcus uberis*, and *Klebsiella pneumonia* typically occurs between milkings. These pathogens are found in the environment of the animal, and fecal-contaminated bedding is a common source of exposure to the bacterium. For a short time post-milking, the sphincter muscle at the end of the teat remains open, and if the animal lies down shortly after milking, this may provide an entry way into the udder for bacteria in the soiled bedding (Oviedo-Boyso et al., 2007).

The inflammatory response generated following pathogen entry into the gland can differ between environmental bacteria such as *E. coli* and contagious bacteria like *S. aureus*. Infections due to *E. coli* are typically characterized by acute inflammation occurring between 12 - 24 hours post-infection with a dramatic increase in the levels of pro-inflammatory cytokines, milk SCC, and permeability of the blood-milk barrier of the mammary gland due to the recruitment of neutrophils into the infected quarter (Petzl et al., 2008, Suojala et al., 2008). Although a majority of *E. coli* infections are cleared in a matter of days, some strains of *E. coli* have been known to cause a more persistent mammary infection lasting up to 30 days in some animals (Fairbrother et al., 2015). In contrast, mammary infections due to *S. aureus* often develop into a chronic condition, and if the infected animal is not removed from the herd, it can serve as a reservoir of the bacteria on the farm (Bannerman et al., 2004). The inflammatory response generated during a *S. aureus* infection is typically minimal or delayed in comparison to *E. coli* infections (Petzl et al., 2008). However, there is evidence that infections with some

strains of *S. aureus* will result in a more severe inflammatory response comparable to that of *E. coli* infections (Peton and Le Loir, 2014).

Concentrations of two cytokines that play a major role in promoting the inflammatory and febrile responses, interleukin (IL) -1β and tumor necrosis factor alpha (TNF-α), reach high levels during a case of *E. coli* mastitis (Bannerman et al., 2004). While a greater TNF-α response during *E. coli* mastitis has been associated with a more severe systemic response in dairy animals (Ohtsuka et al., 2001), levels of TNF-α in milk are often undetectable during *S. aureus* mastitis (Riollet et al., 2000, Bannerman et al., 2004). Irrespective of the pathogen causing the infection, a desirable outcome from a case of mastitis is an effective inflammatory response that clears the infectious agent along with any cellular debris, followed by a quick return to a healthy, functioning gland. As the innate immune system is responsible for the initial recognition of the invading pathogen, this response is effectively the first line of defense against mastitis pathogens once they have evaded the physical barriers of the teat end and entered the mammary gland.

1.3 Innate Immunity and Pathogen Recognition

The innate immune response plays a key role in the defense against infections. This response is initiated within minutes following pathogen exposure and is mediated by macrophages, neutrophils, natural killer cells, and cytokines (Oviedo-Boyso et al., 2007). It has been suggested that the rapidity and magnitude of the innate immune response can influence the progression and resolution of a mammary infection (Bannerman, 2009). Pathogens are recognized by germ-line encoded receptors located either on the

extracellular surface or within the endosomal membrane on multiple cell types within the host (Takeuchi and Akira, 2010). These receptors, known as pathogen recognition receptors (PRR), will recognize and bind to highly conserved molecules referred to as pathogen associated molecular patterns (PAMP) from a variety of pathogens (Takeuchi and Akira, 2010). One class of PRR are the Toll-like receptors (TLR), and there have been 10 TLRs discovered in the bovine (Menzies and Ingham, 2006).

The recognition of lipopolysaccharide (LPS) from gram-negative bacteria by TLR4 requires several adaptor proteins that facilitate the interaction between the receptor and LPS. Small amounts of LPS are released during bacterial replication; however, higher concentrations are liberated following the death and destruction of the bacteria (Zhang et al., 1998). This free LPS is collected by lipopolysaccharide binding protein (LBP), an acute phase protein that has a high affinity for LPS in the bloodstream and the mammary gland (Bannerman et al., 2003, Takeuchi and Akira, 2007). The formation of the LBP-LPS complex allows the trafficking of LPS to a coreceptor that is expressed on the cell surface, cluster of differentiation 14 (CD14), which will then chaperone the LPS to an accessory molecule, myeloid differentiation factor 2 (MD2) that is attached to the extracellular portion of TLR4, initiating a downstream signaling cascade (Mogensen, 2009).

TLR2 recognizes a diverse repertoire of molecules from gram-positive bacteria, including lipoproteins, peptidoglycan, and lipoteichoic acid (Takeuchi and Akira, 2007). This TLR has the ability to form a heterodimer with either TLR1 or TLR6, thereby expanding the number of ligands that can be recognized (Takeuchi and Akira, 2007). Cluster of differentiation (CD) 36, a transmembrane glycoprotein with several biological

roles including platelet function and angiogenesis, has been shown to be an important co-receptor of TLR2 (Jimenez-Dalmaroni et al., 2009). However, the requirement for CD36 seems to be ligand-dependent, and CD36 specifically facilitates the sequestering and recognition of bacterial diacylglycerides by TLR2/6 heterodimers (Hoebe et al., 2005, Jimenez-Dalmaroni et al., 2009). Additionally, the adaptor protein CD14, which is required for recognition of LPS by TLR4, has been shown to facilitate the recognition of several ligands derived from gram-positive bacteria by TLR2 (Wetzler, 2003, Janot et al., 2008).

Following binding of the respective ligand by a TLR, two different intracellular pathways can be activated, one utilizing the adaptor protein myeloid differentiation primary response gene 88 (MyD88), and the other employing the protein Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF) (Mogensen, 2009). Within the ten TLRs in the bovine, every TLR utilizes the MyD88-dependent pathway except for TLR3, which instead signals through the TRIF-dependent pathway, and TLR4, which has a unique ability to signal through both the MyD88-dependent and the TRIF-dependent pathways (Mogensen, 2009, Kawai and Akira, 2010). Factors that may influence which pathway is followed after LPS recognition include the bacterial species the LPS originated from, variations in the conformation and structure of the lipid A moiety of the LPS, and the amount of the various adaptor proteins available to assist in the recognition of LPS (Zughaier et al., 2005).

Activation of the MyD88-dependent pathway will promote the phosphorylation and degradation of the inhibitory protein IκB, freeing the transcription factor nuclear factor-kappa B (NF-κB) and allowing its translocation into the nucleus to promote

transcription of various inflammatory genes (Takeuchi and Akira, 2010). Simultaneously, mitogen activated protein kinases (MAPK) will be activated, and induce the activity of a second transcription factor, activator protein 1 (AP-1) that also promotes the expression of various cytokines. The TRIF-dependent pathway results in the phosphorylation, activation, and nuclear translocation of the transcription factor interferon regulatory factor 3 (IRF3), which is required for the induction of Type I interferons (IFN) and IFN-inducible genes. Additionally, the TRIF pathway does lead to a delayed activation of NF-κB that contributes to the later-phase of the inflammatory response (Mogensen, 2009).

Each of the TLR signaling pathways lead to the activation of the transcription factor NF- κB, which along with AP-1 and IRF3, control the expression of various pro- and anti-inflammatory genes (Kawai and Akira, 2007). Upon nuclear translocation of NF- κB, production of cytokines such as IL-1β, TNF-α, and IL-6 along with chemokines such as IL-8 and chemokine (C-C motif) ligand 5 (CCL5) is stimulated (Kawai and Akira, 2007). Cytokines are small soluble proteins that have the capacity to influence multiple biological processes within the body (Dinarello, 2007). IL-1β and TNF-α are two key pro-inflammatory cytokines whose effects are similar: promoting a fever, activating endothelial cells and leukocytes, and inducing a systemic acute phase response (Bannerman, 2009). Due to the overlapping roles these two cytokines play in the inflammatory response, high levels of both cytokines during a case of mastitis can result in deleterious effects including systemic shock, blood vessel leakage, and in severe cases, multiple organ failure and death (Bannerman, 2009). Similarly, IL-6 can induce fever, lymphocyte activation, and increase the magnitude of the inflammatory response by inducing the synthesis of acute phase proteins by the liver. Higher levels of IL-6 as well

as IL-1β in milk from dairy cattle suffering from mastitis have been correlated with a greater severity in clinical symptoms (Hagiwara et al., 2001, Bannerman, 2009).

Chemokines are a large family of cytokines that derive their name from their chemotactic ability. These proteins are produced from a variety of cells and recruit various types of immune cells including antigen-presenting cells, neutrophils, and lymphocytes to sites of infection (Sokol and Luster, 2015). Expression of chemokines such as IL-8, CCL20, and CCL5 will increase dramatically in the infected mammary tissue, stimulating the influx of neutrophils, lymphocytes, and monocytes, respectively (Bannerman, 2009). During mastitis, neutrophils are recruited into the gland from the blood due to elevated levels of IL-8 in tissue and milk. These cells will make up approximately 90% of the incoming immune cell population and are the primary phagocytic cells entering the mammary gland during the early phase of the infection (Sordillo and Streicher, 2002).

An effective, yet controlled inflammatory response is needed in order to maximize the clearance of the pathogen while minimizing collateral damage to the mammary gland during mastitis. The magnitude of the innate immune response can be quite variable between animals during a case of mastitis, which can influence the progression and resolution of the infection.

1.4 Phenotypic Variation in the Innate Immune Response

The infecting pathogen or differences in endogenous host physiology can influence the magnitude of the innate immune response following bacterial mastitis. As discussed above, the response generated towards *E. coli* and *S. aureus* mammary

infections are typically quite different. However, host factors can also influence the initiation and progression of the inflammatory response towards mastitis (Burvenich et al., 2003). This is best demonstrated in experimental mammary challenge models that control for pathogen factors, such as the strain and amount of bacteria infused. Schukken et al. (1999) observed a large degree of variation in the propensity for an animal to become infected following an experimental quarter-level *S. aureus* challenge conducted on 135 dairy cows, leading to the conclusion that individual cow factors led to the inter-animal differences in the tendency to develop an infection. Additionally, a large range in the extent of clinical symptoms and bacteria clearance rates was present in 36 cows that underwent an intra-mammary challenge with *E. coli* (Kornalijnslijper et al., 2003).

The clinical symptoms during a natural exposure case of bovine mastitis can be variable as well, and these symptoms, regardless of the infecting pathogen, can be grouped into a three-grade severity scoring system. This system is broken down into mild cases, which have minor changes in milk consistency but no systemic signs, moderate cases that include localized swelling and discomfort of the mammary gland along with flakes or clots in the milk, and severe cases of mastitis with acute swelling of the mammary gland, changes in milk consistency and/or color, and a systemic inflammatory response (Pinzon-Sanchez and Ruegg, 2011). Animals can exhibit a range of clinical symptoms during natural mastitis caused by a variety of bacteria, as demonstrated by a survey conducted on 50 Wisconsin dairy farms (Oliveira et al., 2013). Within the mastitis cases that had a severity score recorded, 47.8, 36.9, and 15.3% had clinical symptoms that were classified as mild, moderate, and severe, respectively. Furthermore, a majority

of the severe cases of mastitis observed on these Wisconsin dairy farms were due to *E. coli* or other gram-negative bacteria (Oliveira et al., 2013).

These studies highlight that the susceptibility to mastitis and the resulting inflammatory response are quite variable between animals. While some animals respond with a low degree of inflammation and suffer few, if any, clinical symptoms, other animals will mount a more robust response, characterized by high levels of cytokines and chemokines that could inadvertently inflict collateral damage to the mammary tissue (Zhao and Lacasse, 2008, Schukken et al., 2011). There has been much debate as to what type of response is better for pathogen clearance and the host: a rapid, more robust response or a slower, more moderate one.

Through the use of an autologous dermal fibroblast model, the innate immune response of dairy cows towards an intra-mammary *E. coli* challenge was predicted (Kandasamy et al., 2011). Animals were classified as a low or high responding phenotype based on the production of IL-8 from their fibroblast cultures following a challenge with LPS, and animals from the top and bottom 15% of the range of IL-8 responses were selected for the intra-mammary challenge. It was observed that animals that were ranked as high responders exhibited greater levels of tissue damage within the mammary gland, determined by higher levels of bovine serum albumin (BSA) in milk, as well as a greater influx of neutrophils into the infected gland during the resolution phase of the infection (determined by SCC). Interestingly, the greater recruitment of neutrophils into the gland of the high responding animals did not result in faster *E. coli* clearance, as these animals cleared the infection in a similar time frame (approximately 7 d) as the low responders. This suggests that the heightened inflammation generated by the high responding animals

in response to the *E. coli* challenge did not offer any advantage in the resolution of the infection. Additionally, animals with a robust inflammatory response are at a greater risk for suffering collateral tissue damage during mastitis, increasing the potential for a long-term reduction in milk yield from that animal.

1.5 Breed Differences in the Innate Immune Response

Cattle, as a species, have undergone dramatic selection pressures from producers in order to select for desirable traits, such as milk production or feed efficiency. As a result of this selection, several different breeds of cattle have arisen; each with certain distinguishable characteristics, and these cattle breeds can be grouped into two sub-species, *Bos indicus* or *Bos taurus*. Cattle breeds of the *B. indicus* sub-species show better thermotolerance and can better utilize poorer quality forages compared to *B. taurus* breeds, however, *B. taurus* breeds typically produce greater volumes of milk and show greater lactation persistency.

It has been shown that *B. indicus* breeds, such as the Brahman and Sahiwal, have greater resistance to tick infestations and tick-borne diseases as compared to *B. taurus* breeds like the Holstein (Glass et al., 2005, Piper et al., 2008). During an experimental tick infestation, Brahman animals had a lower number of attached ticks than Holsteins (15 vs. 151 per side of the animal, respectively) (Piper et al., 2008). Skin biopsies collected from both the Holsteins and Brahmans at tick attachment sites demonstrated that Holsteins had greater expression of many innate immune response-related genes, such as MyD88, CD14, TRAF-6, IL-1β, and TNF-α, compared to the Brahmans. Furthermore, Holsteins exhibited a more severe clinical response to a *Theileria annulata*

challenge, the causative agent for the tick-borne disease, tropical theileriosis, resulting in the euthanasia of all Holsteins prior to the conclusion of the trial. In contrast, the Sahiwals seemed to be more resistant to the deleterious inflammation and recovered from the challenge, clearing the parasite by day 14 post-infection (Glass et al., 2005). These studies seem to suggest that the Holstein breed has a more vigorous innate immune response compared to *B. indicus* breeds, and that this may not be beneficial.

The breeds of the *B. taurus* sub-species can be further broken down into dairy cattle (selected for high volumes of milk production), beef cattle (selected for calf rearing ability and feed efficiency), and dual purpose cattle (selected for traits of both dairy and beef animals). Several studies have examined the innate immune response of various *B. taurus* breeds as the selection pressures imparted on these breeds are quite different, depending on the desirable traits the producer is selecting for. Intra-mammary challenges completed on Holstein and Jersey cattle, the two most common dairy breeds within the United States, revealed that the innate immune response towards mammary infections due to *E. coli* (Bannerman et al., 2008a) or *S. aureus* (Bannerman et al., 2008b) is relatively conserved between these two breeds. Additionally, following an intravenous LPS challenge on Angus and Romosinuano beef cattle, differences were observed between the breeds in levels of inflammatory mediators (Carroll et al., 2011). Although Angus steers experienced a higher serum cortisol response, Romosinuano steers had greater levels of serum TNF-α and IL-1β along with a higher febrile response following the LPS challenge. The author concluded that the Romosinuano breed seem to mount a more robust response towards LPS while the heightened cortisol response of the Angus

animals suggested that this breed may be better able to control the inflammatory response.

There is a surprising lack of studies that have explored differences between dairy and beef breeds, particularly in regards to mastitis susceptibility. Several groups have collected milk samples from beef and dairy animals to determine rates of mastitis and the bacterial species causing infections. A mastitis survey was completed on ten beef herds consisting of Herefords, Simmentals, and Limousin breeds, in which milk samples were collected during the month after calving and again at weaning (Persson Waller et al., 2014). Averaged across the herds, 40% of the cattle experienced a mammary infection at calving or weaning, and the majority of these infections were caused by *S. aureus*, followed by coagulase negative *Staphylococcus* species. Surprisingly, there were no coliform species isolated from any of the beef animals. This lack of coliforms or other gram-negative bacteria isolated from quarters of beef cows has been documented in other studies (Paape et al., 2000, Lents et al., 2008). This observation is surprisingly different from mastitis surveys completed on dairy herds, during which the rate of coliform isolation from milk samples ranges between 10 - 35.6% (Oliveira et al., 2013, Hertl et al., 2014, Levison et al., 2016). The apparent lack of coliform mastitis in beef animals may indicate genetic or epigenetic breed differences that impart a degree of resistance to these pathogens by these breeds. However, differences between typical environments of lactating beef and dairy animals, such as lower stocking density and more frequent suckling in beef animals, may also affect mastitis incidence.

Based on these findings, it may be of interest to investigate potential differences in the innate immune response between adult dairy and beef animals under controlled

environmental conditions. These animals are selected for different traits, which may lead to genetic differences in the immune response between beef and dairy cattle. Additionally, the young are reared quite differently between beef and dairy herds. Dairy calves are separated from the dam at birth and fed a milk-replacer based diet for the first 6 - 7 weeks of life. In contrast, beef calves remain with the dam out on pasture, and are allowed to nurse for the first 3 - 6 months of its life. These differences in the early-life environment of dairy and beef calves may cause epigenetic modifications that could change the expression of innate-immune response genes, thereby influencing disease resistance or severity in the adult animal.

1.6 Epigenetics

A phenotypic trait of an animal results from the combination of that individual's genetic make-up and how the environment has influenced the expression of genes. Although mutations within the DNA sequence can cause changes in gene expression between animals (Novak, 2014), some modifications induced by the environment do not change the primary DNA sequence. These are termed epigenetic modifications, and epigenetics is the study of these alterations, and how they impact gene expression and the animal's phenotype. Epigenetic changes can include histone acetylation and methylation, long non-coding RNAs, and DNA methylation (Breiling and Lyko, 2015, Larriba and Del Mazo, 2016, Messier et al., 2016). While these modifications are responsible for the regulation of several biological processes, including cell differentiation (Alvarez-Errico et al., 2015), X chromosome inactivation in females (Engel, 2015), and aging (Munoz-Najar and Sedivy, 2011); aberrant epigenetic changes have been linked to human diseases

such as Huntington's (Glajch and Sadri-Vakili, 2015), Alzheimer's (Sanchez-Mut and Graff, 2015), and several types of cancer (Gu et al., 2015, Vizoso et al., 2015).

1.7 DNA Methylation

DNA methylation is a covalent epigenetic modification that involves the addition of a methyl group onto the 5th carbon within the 6-carbon ring of the nucleotide cytosine. This "classic" epigenetic mark most often occurs at cytosine-guanine (CpG) dinucleotides, and is associated with transcriptional silencing of the gene (Schubeler, 2015). A group of enzymes known as DNA methyltransferases (DNMTs) mediates the addition and maintenance of the methylation pattern on the cytosines. DNMT1, the maintenance enzyme, has a high affinity for hemimethylated strands of DNA following DNA replication, and will copy the methylation pattern from the parent to the daughter strand. DNMT3A and 3B are the de novo methyltransferases that establish the methylation pattern during early development (Uysal et al., 2015).

The pattern of DNA methylation is dynamic during an animal's lifetime, gaining and losing methylation at critical points in development or during cellular reprogramming. There has been much work in determining the mechanisms involved with the removal of methylation from DNA. One proposed method is the down regulation of the DNMTs, resulting in a gradual reduction in DNA methylation with each cell division. A second method that was recently discovered represents a more active demethylation that is catalyzed by a family of enzymes known as ten-eleven translocation (TET) proteins (Piccolo and Fisher, 2014). This is accomplished through a series of oxidation steps that result in the conversion of 5-methyl cytosine (5mC) to 5-

formylcytosine (5fC) and 5-carboxylcytosine (5caC). These altered bases will then be removed by the enzyme thymine-DNA glycosylase, and base excision repair machinery will insert an unmodified cytosine nucleotide (He et al., 2011, Rasmussen and Helin, 2016).

Several high throughput-sequencing techniques have been developed to examine the methylome of an individual on a genome wide scale, each with distinct strengths and weaknesses. Within the bovine research field, whole genome bisulfite sequencing, reduced representation bisulfite sequencing (RRBS), and techniques that depend on the selective binding and precipitation of methylated DNA are available. Selection of one technique over another depends on the economic feasibility, the amount of sample available, and the desired sensitivity of the end result.

During whole genome bisulfite sequencing, the genomic DNA is treated with sodium bisulfite, which will change unmethylated cytosines to uracil by a process known as deamination, while methylated cytosines are protected (Clark et al., 2006). After the conversion, the products are sequenced, allowing for the comparison of converted to unconverted DNA from each sample. Although this technique is considered the gold standard in determining the methylome of an individual at base pair resolution, it requires a tremendous amount of sequencing per sample, making the cost of whole genome bisulfite sequencing prohibitive for many researchers.

A second technique that is largely based off of whole genome bisulfite sequencing is RRBS. This technique provides a cheaper alternative for researchers interested in mainly the methylation status of CpG sites in gene promoters and other regions of the genome with high CpG content. The genomic DNA is first treated with the restriction

enzyme Msp1, which cleaves at 5'-C^CGG-3' sites. The resulting DNA fragments are then size-selected to lengths appropriate for sequencing, and treated with sodium bisulfite to convert the unmethylated cytosines to uracils. These fragments will have a CG at one end, as well as potential internal CGs. Following PCR amplification and sequencing, the sequences are compared to a whole genome reference sequence, such as Bos-taurus-UMD-3.1, to determine methylated CpG sites (Gu et al., 2011). The amount of sequencing following the bisulfite conversion in RRBS is substantially less than that of whole genome, reducing the overall costs and making RRBS a more economically permissive technique for many researchers.

Analysis of DNA methylation may also be completed by the creation of DNA libraries following enrichment of methylated regions of DNA. This can be accomplished with either an antibody-based method or a technique that uses a complex of proteins that bind methylated DNA. Methylated DNA immunoprecipitation sequencing (MeDIP-seq) utilizes antibodies generated against either 5mC or 5-hydroxymethylcytosine (5hmC), an oxidation product of the demethylation of 5mC by TET proteins, to precipitate methylated DNA fragments that can then be sequenced. The DNA does not need to be treated with sodium bisulfite; however, it must be denatured in order to allow for efficient binding of the antibodies to 5mC or 5hmC (Fouse et al., 2010). Methylated-CpG Island Recovery Assay (MIRA) works by capitalizing on the high binding affinity that the complex formed by methyl-binding protein MBD2b and its homologue, MBD3L1 has towards methylated DNA (Jung et al., 2015). This technique does not require denaturation or sodium bisulfite treatment of the DNA, and the MBD proteins do not interact with 5hmC, thus allowing for specificity towards 5mC (Jin et al., 2010).

Although MIRA and MeDIP-Seq are relatively inexpensive techniques to determine DNA methylation patterns of an animal's genome, neither technique offers single base-pair resolution as bisulfite-sequencing methods do.

Lastly, a technique that is currently only available for human research is the Illumina 450k Methylation Bead Chip that examines over 480,000 CpG sites within the human genome following sodium bisulfite treatment of the DNA (Ma et al., 2013). Released in 2011, this array targets each of the selected CpG sites by two different probe sets: one that matches the methylated sequence and that matches the unmethylated sequence. While this technique can provide a researcher with the methylation status of several CpG sites, this array covers only 1.7% of the total CpG sites within the human genome (Ma et al., 2013).

1.8 Aberrant DNA Methylation and the Inflammatory Response

As discussed previously, DNA methylation is a key modification that regulates several biological processes within the body. However, a growing body of evidence demonstrates that differences in methylation pattern can result in differences in gene expression that may influence the magnitude of the inflammatory response generated towards a pathogen. Several factors have been shown to induce methylation differences, including bacterial and viral infections, and inflammation associated with those infections.

Bacteria are capable of inducing epigenetic modifications within the host that can create an environment more suitable for the bacteria's survival. *Porphyromonas gingivalis*, the predominate bacteria involved in chronic periodontitis, induces an increase

in methylation within the TLR2 promoter of gingival epithelial cells, reducing TLR2 expression and thereby modulating the host's innate immune response (Benakanakere et al., 2015). In some instances it is not the pathogen directly but rather the inflammation associated with the infection that induces the changes in DNA methylation. During experimental chronic infections with *Helicobacter pylori* in gerbils, the level of DNA methylation in intestinal epithelial cells increased and it was determined that the inflammatory response associated with the infection led to the greater methylation (Niwa et al., 2010). However, not every alteration in methylation is associated with a disease state. As compared to pathogens, commensal bacteria within the intestines induce greater methylation of the TLR4 gene promoter of intestinal epithelial cells, reducing its expression and preventing an excessive inflammatory response towards the "good" bacteria (Takahashi et al., 2011).

Cytokines play a key role in the initiation and progression of inflammation following pathogen exposure, and in some diseases, an exacerbated inflammatory response can result in severe collateral damage to the host. Elevated levels of serum IL-6 and IL-8 in human patients suffering from inflammatory rheumatoid arthritis and severe periodontitis have been linked to lower methylation in the promoter regions of those genes as compared to healthy controls (Andia et al., 2010, Ishida et al., 2012). In another example, the dengue virus is a prevalent arthropod-borne virus that in some patients can cause a severe hemorrhagic form of dengue fever characterized by over-expression of TNF-α. In these patients, the levels of methylation within the TNF-α gene promoter are lower compared to other individuals that developed the more moderate form of disease. This suggests that the reduced methylation levels of the TNF-α promoter could be

associated with the heightened TNF-α expression during this disease (Gomes et al., 2016). Additionally, Sun et al. (2013) observed that while the expression and production of interferon-gamma (IFN-γ) is absent in newborn foals, it increases with age. This age-dependent increase in IFN-γ production was determined to be due to a gradual loss of methylation within the IFN-γ promoter. While reduced IFN-γ production may be a protective strategy to prevent fetal loss in utero, following birth, the IFN-γ production must increase quickly in order to protect the foal against intracellular bacterial infections.

Changes in DNA methylation have been linked to an increased risk for several human diseases, including those associated with a dysregulation of inflammation and various types of cancer. While genetic factors can lead to the development of non-alcoholic fatty liver disease (NAFLD), some individuals develop a more severe form of the disease and this variation cannot be explained by genetics alone. A mouse model revealed that the levels of DNA methylation within the liver pre-determined an individual's propensity towards developing the more severe form of NAFLD (Tryndyak et al., 2016). Likewise, an asthma study completed on a cohort of monozygotic twins uncovered that differences in the development of asthma was due to functional changes of T cell subsets associated with DNA methylation differences between the twins (Runyon et al., 2012). Additionally, aberrant methylation as well as other epigenetic modifications of several cytokine genes have been associated with their transcriptional dysregulation, leading to an increased risk for the development of several types of cancer, including breast, cervical, gastric, colorectal, and pancreatic (Yasmin et al., 2015).

Within the field of bovine research, epigenetic modifications and the role they may play in susceptibility to disease is a recent area of study. Still, several studies have

revealed key aspects regarding DNA methylation and the modulation of the immune response in the dairy animal. Through the use of a dermal fibroblast cellular model, Green and Kerr (2014) demonstrated that DNA methylation was at least partially responsible for an age-dependent increase in the IL-8 response following LPS exposure of fibroblasts isolated from the same animals at 5 and 16 months of age. Additionally, Wang et al. (2013b) determined that the levels of methylation in the promoter region of the CD4 gene in whole blood were 16% higher in mastitic cows than healthy cows. The increased promoter methylation was associated with a reduced expression of CD4 that may have been due to the methyl groups prohibiting binding of transcription factors in the affected animals. In an experimental mastitis model, liver biopsies were collected from dairy animals that had undergone an intra-mammary *E. coli* challenge to determine epigenetic mechanisms that may contribute to the initiation and progression of inflammation. Chromatin analysis on four innate immune response genes, TLR2, TLR4, LBP, and haptoglobin (Hp) revealed that the increased expression of these genes in the challenged animals compared to healthy controls was associated with opening of the chromatin. Additional analysis on the TLR4 gene revealed the *E. coli* infection induced a loss of promoter methylation, leading to its greater expression (Chang et al., 2015).

1.9 Fetal and Early Life Programming

The innate immune response of an animal can be influenced by events occurring to the dam during pregnancy, and during the neonatal period. Aspects of the maternal diet can induce changes within the in utero environment, which can modulate features of the offspring's immunity. Late pregnant cows are often supplemented with mineral mixes to

ensure an adequate supply of minerals for fetal growth and parturition. Traditionally, cows have been fed an inorganic sulfate-based mineral mix, but new mineral mixes have been developed that contain chelates, or trace minerals complexed with amino acids and other molecules to increase their absorption by the cow (Swecker, 2014). There is some evidence that the chelate mixes also promote an enhanced immune response around parturition (Wang et al., 2013a), however, the effects of mineral mix supplementation on the calf are unknown. Therefore, groups of pregnant cows were fed either a sulfate-based or a chelate mineral mix for the last 30 days of their pregnancy and the expression of innate-immune response genes were examined in neutrophils isolated from their calves at birth, and 1, 7, and 21 days of age (Jacometo et al., 2015). Neutrophils from calves whose dams were fed the sulfate-based minerals had greater expression of several genes within the toll-like receptor pathway, including MyD88, IRAK1, TRAF6, and NF-κB, as well as increased expression of micro RNA (miR) 155 as compared to those calves from dams fed the chelate mix. These results reveal that mineral supplementation as well as the specific formulation of the mineral supplied to the dam can affect the expression of immune response genes within the calf. Although the increased expression of miR-155 was suggested as a possible mechanism responsible for inducing the greater gene expression in calves from sulfate-based mineral supplemented dams, epigenetic modifications induced by the maternal mineral mix supplementation may also play a role.

Obesity has been shown to induce a pro-inflammatory state within the intestines, which may be linked to the development of inflammatory bowel disease (IBD). A sheep model was used to determine the effects of maternal obesity on the inflammatory gene response within the intestines of the offspring and the likelihood to develop IBD (Yan et

al., 2011). Non-pregnant ewes were fed an obesogenic diet from 60 days prior to conception till lambing, and intestinal biopsies were collected from lambs at birth and 22.5 months of age for mRNA analysis. Within the intestines of the offspring of obese dams, there was greater expression of TLR2, TLR4, IL-6, IL-8, and transforming growth factor beta (TGF-β), as well as greater fibrosis of the intestinal lining compared to offspring from dams fed a control diet. As TGF-β and IL-6 have been shown to drive the differentiation of Th17 cells, a population of inflammatory T-cells that play a crucial role in the development of IBD, it was suggested that the greater expression of these two genes and other pro-inflammatory mediators led to an increase in Th17 cells in the intestines, further promoting inflammation. As these increases in gene expression and fibrosis were not observed in the control lambs, it was determined that feeding an obesogenic diet to the dam during pregnancy was the primary factor driving the increased inflammation.

Maternal inflammation due to an infection can lead to the production of inflammatory mediators that can affect the placenta or produce a febrile response that could impact the growing fetus. Several studies have utilized an injection of LPS given to the dam to induce maternal inflammation and determine its effects on the offspring. A single prenatal injection of LPS administered to rats at day 12 of pregnancy resulted in decreased levels of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH). This created an imbalance in the levels of sex steroid hormones in both male and female offspring, and delayed the sexual maturation in the females (Izvolskaia et al., 2016). Additionally, Depino (2015) reported that adult mice whose dams had received a single injection of LPS at day 9 of gestation experienced greater anxiety-like behaviors as

compared to adult mice whose dams were treated with saline. In another study, LPS was administered to pregnant mice at the stage of zygote formation (day 0.5 of pregnancy), and the offspring of these dams underwent a subsequent LPS challenge in adulthood (Williams et al., 2011). Adult mice born to dams that had received LPS displayed a blunted cytokine response following the subsequent LPS challenge compared to the offspring of saline control dams.

Furthermore, immediately after birth the offspring is exposed to a myriad of bacteria and environmental factors that could lead to modifications of that individual's immune response phenotype. To mimic an early life exposure to bacterial infection, but avoid the bacterial replication, LPS is a common compound used to induce an inflammatory response in early life exposure studies. Mouihate et al. (2010) observed that rats exposed to LPS on day 14 of life experienced a reduction in the magnitude of the overall immune response within the brain towards a subsequent LPS challenge given in adulthood. Similarly, neonatal female mice that received an injection of LPS at days 3 and 10 exhibited a reduced IL-6 and TNF-α response towards an inflammatory stimulus given both during adolescence (21 days of age) and adulthood (60 days of age) (Barth et al., 2016). Another compound that has been shown to induce changes in gene expression in the lung epithelium is ozone. A postnatal exposure to ozone in rhesus macaque monkeys resulted in a decrease in IL-6 mRNA and protein from lung epithelial cells following an in vitro LPS challenge. It was determined that the reduced IL-6 expression and protein was mediated by increased expression of miR-149, a micro-RNA that binds to the 3' UTR of the IL-6 gene and inhibits transcription (Clay et al., 2014).

While the mechanisms that lead to these modifications in disease and inflammation susceptibility have not been fully elucidated in livestock, several human and mice studies have revealed a key mechanistic role for epigenetic modifications. A recent review by Vaiserman (2015) described several maternal factors that can alter the epigenome of the human fetus. For example, maternal stress and depression during the prenatal period led to greater methylation and a reduced expression of the fetal glucocorticoid receptor gene. Additionally, exposure to cigarette smoke during pregnancy results in the alteration of methylation patterns and the dysregulation of the expression of multiple miRNAs that have been implicated in growth and development. Glucocorticoid is often administered if a pre-term delivery is expected to aid in the development of the fetal lung. However, the impact of the glucocorticoid treatment on the infant's immune system has not been well defined. Pregnant rats were given dexamethasone from days 14-20 of gestation, and male pups were used to determine the effects of dexamethasone on the immune response. Levels of TNF-α mRNA were reduced in the spleens of 7 and 120 day old rats which had been exposed to prenatal dexamethasone compared to that of saline controls. A chromatin immunoprecipitation assay (ChIP) revealed a loss of active chromatin marks (acetylation of histone H3 lysines) in the TNF-α gene promoter of spleens from rats that had been exposed to maternal dexamethasone (Yu et al., 2014).

A concept within human research that seeks to explain the increased disease risk over the lifetime of an individual is the Developmental Origins of Health and Disease theory. Essentially, the idea is that harmful exposures in the early life of an individual can lead to genetic reprogramming that may have long-term consequences on the development of subsequent diseases (Heindel et al., 2015, Heindel and Vandenberg,

2015). The exact outcomes of these insults can vary, depending on the magnitude, timing, and duration of the insult itself, as well as genetic background of the exposed individual. It has been shown that some of these insults can cause epigenetic modifications that cross generations, especially if the insult occurs at a crucial stage of primordial germ cell development. However, continued research is required, especially within the field of livestock research to determine long-term effects on the offspring that can be induced by maternal disease, vaccination, diet, management, and other factors.

1.10 Hypotheses and Specific Objectives

The innate immune response plays a critical role in the initiation, progression, and resolution of inflammation during a mammary gland infection. Within the dairy cattle population, a wide range exists in the magnitude of the innate immune response generated towards bacterial mastitis. While some animals mount a robust response characterized by heightened cytokine levels and collateral tissue damage, other animals seem better able to minimize the inflammation yet still mount an effective response to clear the pathogen. The host factors leading to this inter-animal variation have not been fully elucidated, but several studies have indicated that the innate immune response can be influenced by a combination of genetic and epigenetic mechanisms.

This dissertation tests a number of hypotheses:

1. The response of autologous dermal fibroblasts to a TLR2/6 agonist challenge can be used as a cellular model to predict the magnitude of the innate immune response towards an intra-mammary *S. aureus* challenge in dairy cattle.
2. A combination of genetic and epigenetic factors contributes to the observed differences in the LPS response of dermal fibroblasts isolated from Angus and Holstein cattle.
3. Exposure to bacteria, or bacterial compounds in early life can induce epigenetic modifications within the innate immune response genes, and maintenance of these modifications can affect the innate immune response in the adult animal.

Through the utilization of the dermal fibroblast model as well as animal challenge models, it is hoped that this work will further the understanding of the roles that genetic and epigenetic differences play in impacting the variable innate immune response of the dairy cow. Based on evidence from previous bovine and rodent studies, our specific objectives are:

Experiment 1:

To examine differences in the innate immune response and propensity to develop a chronic infection following an intra-mammary *S. aureus* challenge on two groups of cattle that were ranked as "low" and "high" innate immune responders based on their isolated dermal fibroblasts.

Experiment 2:

To determine the causes that led to the contrasting LPS response from dermal fibroblasts collected from Angus and Holstein cattle by performing whole transcriptome analysis to more fully explore gene expression differences and a genome-wide DNA methylation assessment to examine epigenetic differences in cultures from these two breeds.

Experiment 3:

To determine if a single exposure to LPS during the first week of life can induce sustained effects on the innate immune response to a subsequent LPS challenge, and if so, if the altered *in vivo* responses are reflected by *in vitro* differences in gene expression by autologous dermal fibroblasts and monocyte-derived macrophages.

Chapter 2: Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with *Staphylococcus aureus.*

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2.1 Abstract

Staphylococcus aureus is a common cause of chronic mammary gland infections in dairy cattle. However, the inflammatory response and duration of infection following pathogen exposure is variable between individual animals. To investigate interanimal differences in immune response, dermal fibroblast cultures were established from skin biopsies collected from 50 early lactation Holstein cows. The fibroblasts ability to produce IL-8 in response to a 24-h treatment with a synthetic toll-like receptor 2/6 agonist (Pam2CSK4) was used to assign a response phenotype to the animals. Five high-responding and 5 low-responding animals were then selected for an intramammary challenge with *S. aureus* to evaluate differences in the inflammatory response, chronicity of infection, and development of antibodies to the pathogen. All animals exhibited clinical symptoms of mastitis at 24 hours post challenge. Animals previously classified as high responders experienced a greater inflammatory response characterized by elevated levels of milk somatic cell count, IL-8, and BSA following the challenge compared with low responders. In addition, antibodies toward the challenge strain of *S. aureus* reached higher levels in whey from the challenged gland of high responders compared with low responders. Despite the antibody response, all 5 high responders were chronically infected for the 6-wk duration of the study, whereas 2 of the low responders cleared the infection, although 1 of these did become reinfected. The observed differences between animals classified as low and high responders based on their fibroblast responsiveness suggests that this cell type can be used to further examine the causes of interanimal variation in response to mammary infection.

2.2 Introduction

Bovine mastitis can be caused by a variety of pathogens, resulting in variable degrees of inflammation of the infected mammary gland and subsequent decreases in milk production and quality (Ballou, 2012). Infections caused by *S. aureus* typically result in a relatively mild inflammatory response with a sustained elevation in SCC; however, some animals will experience a more severe response, leading to collateral damage of the host (Atalla et al., 2009). These infections can develop into chronic, subclinical cases, in which infected animals maintain a reservoir of *S. aureus* on a farm (Bannerman et al., 2008, Schukken et al., 2011). Rates of mastitis due to *S. aureus* have decreased since the implementation of the 10-point plan from the National Mastitis Council (NMC, 2011), which recommends teat dipping and dry cow therapy as ways to minimize the spread of contagious pathogens. However, *S. aureus* is still present on most commercial herds, with incident rates ranging from 0.1 – 10 % (Barlow et al., 2013, Hertl et al., 2014, Olde Riekerink et al., 2008). Treatment of long-term infections with antibiotics commonly yields an unsatisfactory cure rate (Barkema et al., 2006, Sol et al., 2000), and these animals are typically removed from the herd to limit spread of the pathogen.

Pathogens entering the mammary gland are recognized by toll-like-receptors (TLR), which are found on multiple cell types within the gland and play a pivotal role in the innate immune response. These membrane bound receptors are highly specific and identify conserved motifs known as pathogen-associated-molecular patterns from various pathogens, including bacteria, fungi, and viruses (Takeuchi and Akira, 2010). For instance, LPS, a component of the Escherichia coli outer membrane, activates TLR4

(Park et al., 2009), and lipoproteins from the *S. aureus* cell wall activate TLR2 (Zähringer et al., 2008). Toll-like receptor signaling pathways lead to the activation of the transcription factor nuclear factor-kappa B (NF-κB), which regulates expression of various proinflammatory genes (Kawai and Akira, 2007). Increased expression of chemokines, such as IL-8, following NF-κB activation promotes recruitment of neutrophils to the site of infection. Neutrophils can account for up to 90% of the increased cell population within the infected gland and are the main cause of the rise in SCC following infection (Sordillo and Streicher, 2002).

Pathogen-specific differences in infection outcomes following experimentally induced mastitis and the resulting inflammatory responses have been observed (Bannerman, 2009). However, even in well-controlled situations considerable variation in response exists between individual animals. At the farm level, animals in similar physiological states under common environmental conditions also exhibit variable susceptibility to development of clinical mastitis. Various cell models have been used to investigate potential sources for these interanimal differences, including neutrophils (Sohn et al., 2007; Revelo and Waldron, 2012), monocyte-derived macrophages (Taraktsoglou et al., 2011), and mammary epithelial cells (Lahouassa et al., 2007; Brand et al., 2011). As model cells, neutrophils and macrophages are difficult to cryopreserve for future studies and can become activated during isolation and culturing processes, potentially adding to experimental variation. Mammary epithelial cells show a robust response to immunostimulants such as LPS, and these cells can successfully be cryopreserved (Wellnitz and Kerr, 2004; Pareek et al., 2005). However, although these cells can easily be isolated from tissue collected at euthanasia, procedures to isolate

sufficient mammary tissue from live animals are relatively invasive and may compromise the ability of the gland to return to full production. We have previously examined the autologous dermal fibroblast as a model cell to predict an animal's responsiveness to experimental *E. coli* mastitis (Kandasamy et al., 2011). The fibroblast is easily obtainable and, following isolation, the cells can be cultured quickly and cryopreserved. Cultured fibroblasts also demonstrate a robust response following stimulation with various TLR agonists (Kandasamy and Kerr, 2012), suggesting that it may be a useful cell type to explore the underlying causes for interanimal variation in response to mastitis. In the current study we extend these findings to include experimentally induced *S. aureus* mastitis.

2.3 Materials and Methods

2.3.1 Animal and Experimental Procedures

The University of Vermont's Institutional Animal Care and Use Committee approved all animal procedures before commencement of the study. Dermal fibroblast cultures were established from skin samples collected from 50 lactating Holstein cows housed at a collaborating dairy farm. Animals were randomly enrolled in the study, and at the time of biopsy were in early to mid lactation (117 ± 30 DIM). The average lactation number of the 50 enrolled cows was 2.8 (± 0.9).

Skin samples were collected as described previously (Kandasamy et al., 2011) with slight modifications. Briefly, a 6-cm^2 skin sample was taken from the shoulder area following standard surgical preparation of the site and transported back to the laboratory on ice in Dulbecco's PBS (DPBS; Hyclone Laboratories, Logan, UT) supplemented with

a 1X antibiotic cocktail (100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B; Hyclone Laboratories). After removing subcutaneous fat and connective tissue, a 3-cm^2 piece of skin was minced into smaller pieces with opposing scalpel blades and subsequently washed 4 times with fresh DPBS. After washing, 10 mL of 0.5% collagenase type I enzyme (Life Technologies, Grand Island, NY) diluted in Dulbecco's modified Eagle medium (DMEM; Hyclone) containing 1X antibiotic cocktail was added to the minced skin pieces and incubated at 37°C for 6 h with orbital shaking. The collagenase-digested tissue was then filtered through a 70-µm nylon mesh filter (Fisher Scientific, Pittsburgh, PA), and the filtrate was centrifuged at 1,1000 *x* g for 5 min at 20°C. The cell pellet was reconstituted with DMEM containing 10% fetal bovine serum (FBS; Hyclone Laboratories), 1X insulin-transferrin-selenium (Mediatech Inc., Herndon, VA), and 1X antibiotic cocktail and cultured in a 25-cm^2 flask (Corning Inc., Corning, NY) in a humidified 37°C incubator with 5% CO2 until 70% confluency was reached. Cells were then detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded in a 75-cm^2 flask (Corning Inc.) with DMEM supplemented with 5% FBS, 1X insulin transferrin- selenium, and 1X antibiotic cocktail. After approximately 4 d, cells were trypsinized and split into three 75-cm^2 flasks. Once cells reached confluency, they were lifted with trypsin and aliquots of the third passage were diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and cryopreserved in liquid nitrogen for future challenges.

2.3.2 Dermal Fibroblast Challenges with IL-1β, LPS, and Pam2CSK4

Aliquots of cells were revived from cryopreservation in duplicate and cultured

in a 75-cm^2 flask until confluent. Cells were lifted with trypsin and fourth-passage cells were seeded into a 6-well plate (Corning Inc.) at 1 x 10^5 cells/mL in a total volume of 2 mL. Following a 24-h incubation, media was removed and replaced with 2 mL of fresh media (negative control), or media containing either 1 ng/mL of recombinant bovine IL-1β (AbD Serotec, Raleigh, NC), 100 ng/mL of ultra-pure LPS isolated from *E. coli* 0111:B4 (Sigma-Aldrich), or 200 ng/mL of a synthetic TLR2/6 agonist Pam2CSK4 (PAM2; Invivogen, San Diego, CA). After 24 h of exposure, media was collected from each well, spun at 10,000 x *g* for 1 min to remove cell debris, and stored at −20°C until future analysis.

2.3.3 IL-8 and IL-6 ELISA

The concentration of IL-8 in conditioned media samples was quantified by a custom sandwich ELISA as previously described (Kandasamy et al., 2011). Mouse anti-bovine IL-8 (clone 170.13 from Samuel Maheswaren, University of Minnesota, St. Paul) and biotinylated goat anti-human IL-8 (R&D Systems Inc., Minneapolis, MN) were used as capture and detection antibodies, respectively, and recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) as the assay standard. The concentration of IL-6 in conditioned media was determined with a commercially available bovine IL-6 ELISA kit (Thermo Scientific). The capture and detection antibodies were plated at 1:100 and the streptavidin-horseradish peroxidase was plated at 1:400. Recombinant bovine IL-6 was used as the assay standard (Thermo Scientific).

2.3.4 Preparation of *S. aureus* Inoculum

A cryopreserved *S. aureus* specimen of multilocus sequence type 8, originally

isolated from milk of a cow with chronic subclinical mastitis (Barlow et al., 2013), was streaked on a trypticase soy agar plate with 5% sheep blood (Northeast Laboratory Services, Waterville, ME) and incubated at 37°C for 16 h. One bacterial colony was then used to inoculate 3 mL of trypticase soy broth and incubated at 37°C with shaking for 6 h. Afterward, 1 mL of the inoculated broth was placed in 99 mL of trypticase soy broth and incubated for 16 h at 37°C with shaking. This stock culture was stored for 24 h at 4°C while the bacterial concentration (cfu/mL) was determined by serial dilution and plate counting.

2.3.5 Intramammary Challenge

Ten of the 50 skin-biopsied animals were selected for an intramammary challenge based on the production of IL-8 protein by their dermal fibroblasts in response to stimulation with PAM2. Five of the animals were designated as low responders (LR), as their fibroblast production of IL-8 ranked in the lowest 20% of the 50 animal fibroblast pool, and 5 animals were designated as high responders (HR), as their fibroblast production of IL-8 ranked in the highest 20%. The animals averaged 235 (± 30) DIM and were transported to the University of Vermont 1 wk before the challenge. Four LR and 4 HR animals were confirmed pregnant at the time of challenge (122 ± 34 d pregnant), and controlled internal drug releasers (a progesterone insert, Eazi- Breed CIDR, Pfizer, New York, NY) were placed in the 2 nonpregnant animals to control hormonal fluctuations. Throughout the trial, animals were milked with individual milking units that were sanitized before each milking. Quarter milk samples collected before experimental infection were negative for mastitis pathogens and composite milk SCC were below

200,000 cells/mL. On the day of the challenge, teat ends were disinfected with 70% ethanol and 5 mL of the prepared bacterial suspension diluted in endotoxin-free, isotonic sterile saline was infused with the aid of a teat cannula into the right hind quarter of each animal. Plating of the bacterial infusion solution, followed by colony counting, indicated each animal received 200 cfu in total in the right hind quarter. Milk samples were collected from the infected and control quarters just before infusion and at various times for 6 wk postinfusion (PI). Bacteria counts in milk from individual challenged and control quarters were determined by plating 100 μL of neat or diluted milk samples, incubating at 37°C for 24 h, and counting bacterial colonies. Milk SCC was determined with a portable cell counter (DCC, DeLaval, Tumba, Sweden). Rectal temperatures of each animal were monitored for 4 d PI.

2.3.6 Whey Preparation

Milk samples from the infected and control quarters were centrifuged at 16,000 x *g* for 30 min at 4°C. The fat layer was discarded and the milk was centrifuged a second time at 16,000 x *g* for 30 min at 4°C. The clear supernatant was collected and stored at −20°C for future analysis. Levels of IL-8 protein in whey were determined by ELISA as described previously, and concentrations of BSA in whey were determined using commercial ELISA reagents (Bethyl Laboratories, Montgomery, TX) according to manufacturer directions.

2.3.7 Humoral Response Following *S. aureus* Challenge

Total anti-*S. aureus* IgG antibody levels in plasma and whey were determined by ELISA as described by Leitner et al. (2000) with some modifications. A stock of the

S. aureus challenge strain was prepared as described previously for the intramammary challenge, however, the 100 mL of 16-h growth was subjected to heat-killing at 70°C for 45 min with shaking every 10 min. Aliquots of this stock were stored at −20°C for subsequent assays. Wells of a 96-well ELISA plate were coated with either 100 μL of 1 x 10^7 heat-killed bacteria diluted in 0.05 M bicarbonate buffer or 100 μL of 0.05 M bicarbonate buffer (a blank well) and incubated at 4°C overnight. Plates were washed 3 times with DPBS-0.05% Tween-20 (DPBS-T; Fisher Bioreagents, Fair Lawn, NJ), then 100 μL of a 2% nonfat milk (Lab Scientific, Highlands, NJ) solution diluted in DPBS was added to each well and incubated for 1 h at room temperature. Following plate washing, 100 μL of plasma or whey samples diluted 1:800 or 1:10, respectively, in DPBS-T were plated and incubated at room temperature for 1 h. Plates were washed again and 100 μL of a 0.025- μg/mL solution of a biotinylated polyclonal goat anti-cow IgG (Fisher Scientific), or 100 μL of a 0.2-μg/mL solution of a biotinylated polyclonal sheep anti-bovine IgG1 or IgG2 (Thermo Scientific) diluted in DPBS-T was plated and incubated at room temperature for 1 h. Lastly, after washing, tetramethylbenzidine substrate (Thermo Scientific) was added to each well and the reaction was stopped with 1 M H2SO4 after approximately 2 min. The absorbance at 450 nm was determined using a 4-parameter analysis with optical density correction for blank wells (Synergy-HT, Bio-Tek).

2.3.8 Fibroblast Gene Expression in Response to Pam2CSK4

Gene expression analysis was performed on fibroblast cultures from the animals that were selected for intramammary challenge. Fibroblasts from the 10 challenged

animals (n = 5 LR, n = 5 HR) were revived from cryopreservation, and cultured in a 75-cm2 flask. Once confluent, cells were lifted with 0.25% trypsin, counted, and seeded into 6-well plates at a concentration of 1 x 10^5 cells/mL. Following a 24-h incubation, fibroblasts were exposed to 200 ng/mL of PAM2 and RNA was collected at 3 time points: h 0 (control conditions) and h 2 and 8 post challenge using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer directions. Following extraction, RNA concentrations were assessed using the Qubit 2.0 Fluorometer (Life Technologies). First-strand cDNA synthesis was completed using the Improm II Reverse Transcriptase Kit (Promega, Madison, WI). Expression levels of selected immune genes were determined by quantitative realtime PCR (qRT-PCR) with a 7500 Fastrun Machine (Applied Biosystems, Carlsbad, CA) using Fermentas Maxima SYBR Green/Fluorescein qPCR Mastermix (Thermo Scientific). Messenger RNA expression for target genes was normalized against β-actin, an endogenous control gene. Fold differences in target gene expression are presented as the difference between HR and LR cultures at the indicated hour post-PAM2. Sequences for the constructed oligonucleotide primers are presented in Table 1.

2.3.9 Statistical Analysis

Correlations between IL-8 and IL-6 responses from dermal fibroblasts following 24 h of exposure to IL- 1β, LPS, and PAM2 were calculated by linear regression using the statistical software package Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Differences between production of IL-6 and IL-8 from paired fibroblast biopsies were determined by paired t-test in Prism 6.0. Milk SCC and colony-forming unit counts were

log10 transformed before analysis. Effects of phenotype (LR or HR) on levels of milk IL-8, BSA, SCC, bacteria counts, right hind quarter milk production, and plasma and whey levels of total IgG as well as IgG1 and IgG2 were analyzed with a linear mixed model assuming a first-order auto-regressive covariance structure, with time postinfusion as the repeated measure. Analyses were performed with the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC). Changes in expression of selected immune genes between LR and HR animals were analyzed using a one-tailed t-test. Differences with a $P < 0.05$ were considered significant.

2.4 Results

2.4.1 Stimulation of Dermal Fibroblasts

Isolated fibroblasts from all 50 cows were revived from cryopreservation, cultured, and subsequently challenged for 24 h with PAM2. The concentrations of secreted IL-8 in media following duplicate challenges are presented in Figure 1A. There was a wide range of responsiveness to PAM2, with the mean IL-8 of the highest 10% of animals being more than 8-fold greater ($P < 0.05$) than the mean of the lowest 10% of animals (1243 ± 119 vs. 142 ± 11 pg/mL, respectively). The detection limit of the assay was approximately 130 pg/mL.

The relationships between IL-8 production by the 50 fibroblast cultures following challenges with LPS and IL-1β were also investigated and compared with the response to PAM2 (data not shown). The levels of secreted IL-8 were correlated between the IL-1β and LPS responses ($P < 0.01$, $R^2 = 0.63$), the LPS and PAM2 responses ($P < 0.05$, $R^2 =$

0.46), and the IL-1β and PAM2 responses ($P < 0.01$, $R^2 = 0.80$). To investigate the within-animal stability of the fibroblast response, a second skin biopsy was collected from the 10 animals that had been selected for the in vivo *S. aureus* challenge. This second biopsy was obtained at slaughter on d 40 post challenge, which was approximately 5 mo after the initial biopsy. Unfortunately, 1 of the second HR biopsies did not yield a viable culture due to microbial contamination and was discarded. Fourth passage cultures from the first and second biopsy were revived simultaneously from cryopreservation and challenged in parallel wells with PAM2 for 24 h. The resulting media concentrations of IL-8 and IL-6 are presented in Figure 1B and C, respectively. Responses between replicate biopsies from the same animal were nearly identical for IL-8 with the difference in response magnitude between the LR and HR animals being approximately 4-fold. Responses for IL-6 were also quite similar between the 2 biopsies. The production of IL-8 and IL-6 following PAM2 treatment was highly correlated between replicate biopsies ($P < 0.01$, $R^2 = 0.95$ and $P < 0.01$, $R^2 = 0.75$, for IL-8 and IL-6, respectively).

2.4.2 Rectal Temperature, and Milk Yield, SCC, and Bacterial Counts Following Intramammary Infusion of *S. aureus*

Each of the 10 selected animals received a single intramammary infusion of the challenge strain of *S. aureus* and by 24 h PI, all animals had evidence of mammary swelling and clots in the milk from the challenged quarter. Rectal temperatures in all animals showed a moderate increase that peaked at approximately 30 h PI, with no difference between LR and HR groups (Figure 2).

Milk yield from the right hind quarter (RHQ) is shown in Figure 3A, and although the HR animals produced less milk from the challenged quarter throughout most of the study, this difference was not significant. However, a reduction in quarter milk yield was observed in both groups following the infusion, which reached its lowest value between 48 to 60 h PI.

Immediately before the infusion, milk SCC of each challenged quarter was below 200,000 cells/mL; however, in response to the pathogen, it peaked at approximately 15 x 10^6 cells/mL (Figure 3B). At 24 h PI, the HR SCC (5.2 x 10^6 cells/mL) was approximately 10-fold greater than the LR SCC (3.1 x 10^5 cells/mL), demonstrating a delayed influx of immune cells into the infected gland of the LR animals. Although milk SCC of both groups was elevated above preinfection levels throughout the study, HR animals had higher ($P < 0.05$) SCC in the RHQ milk when compared with LR animals.

Bacterial load was monitored for 6 wk following the intramammary challenge. The challenge strain was recovered from milk samples collected from the RHQ of all animals 24 h after infusion (Figure 3C). One LR animal was culture negative at 84 h PI and maintained culture negative status for the reminder of the study. A second LR animal was culture negative at 108 h PI, but at 336 h PI (d 14) the challenge strain was reisolated from the RHQ and this animal remained infected until the end of the study. Whereas bacterial counts were not significantly different between the 2 groups, none of the HR animals were culture negative during the trial.

2.4.3 Inflammatory Markers Present in Challenged Quarter Whey

The concentration of IL-8 in whey from the RHQ was undetectable until 24 h PI

in both LR and HR groups (Figure 4A). Levels of IL-8 rose quickly after 24 h, however, and HR animals produced a greater ($P < 0.05$) amount of IL-8 following challenge when compared with LR animals. Concentrations of BSA in whey began to increase by 24 h PI and reached a maximum value of almost 3 mg/mL at 72 h PI in the HR animals (Figure 4B). A similar profile was observed in the LR animals, although the rise in BSA was somewhat delayed and the overall magnitude of the response was lower ($P < 0.05$) than in the HR animals.

2.4.4 Plasma and Whey Antibodies to *S. aureus*

Levels of anti-*S. aureus* total IgG in plasma and total IgG, IgG1, and IgG2 in RHQ whey samples were measured by ELISA for 36 d after the challenge. On d 0, the levels of anti-*S. aureus* total IgG in whey and plasma were similar between LR and HR animals (Figure 5A and B). The antibody levels increased in the weeks following the challenge, primarily in the HR animals, such that overall levels of anti-*S. aureus* total IgG were higher ($P < 0.05$) in the HR when compared with the LR animals (Figure 5A and B). Furthermore, this greater response in the HR animals was mirrored by greater increases ($P < 0.05$) in whey levels of the IgG2 isotype of anti-*S. aureus* antibody as compared with LR animals, whereas IgG1 levels were similar between the groups.

2.4.5 Fibroblast Gene Expression in Response to PAM2CSK4

Aliquots of dermal fibroblasts from the 10 animals selected for intramammary challenge were revived from cryopreservation to investigate potential differences in expression of select immune genes in response to PAM2. Total RNA was extracted at h 0 (control conditions), 2, and 8 post treatment. One component of the PAM2 recognition

pathway, TLR2, was induced ($P < 0.05$) by PAM2 stimulation, whereas the other component, TLR6, was not (Figure 6A and B, respectively). Genes associated with an inflammatory response, including IL-8, CCL20, IL-6, and TNF-α, were induced above control levels following PAM2 treatment ($P < 0.05$; Figure 6C, D, E, and F, respectively). These 6 genes were generally expressed at a numerically higher level in the HR cultures than in the LR cultures. Significantly greater expression ($P < 0.05$) in the HR cultures was found for TLR2 and IL-6 at 8 h, and for IL-8 at 0 h post-PAM2. Expression of CCL20 ($P = 0.06$) and TNF-α ($P = 0.11$) at 2 h and TLR6 ($P = 0.06$) at 8 h post-PAM2 showed a trend toward higher levels in HR cultures compared with LR cultures.

2.5 Discussion

In the current study, dermal fibroblasts isolated from adult dairy cattle were used as a cellular model to predict the magnitude of an animal's inflammatory response to an intramammary *S. aureus* challenge. The concept stems from the substantial variation that exists between cows and between cases within cows in the response to mammary gland infection. Differences in pathogen type and strain, differences in farm environment and resulting infection pressure, and differences in physiological state of the animal certainly contribute to variation in incidence and severity of mastitis (Burvenich et al., 2003; Wenz et al., 2006, 2010). However, considerable variation is also observed under controlled experimental challenges with either *E. coli* (Kornalijnslijper et al., 2004) or *S. aureus* (Schukken et al., 1999). In the study by Schukken et al. (1999), 135 lactating cows were challenged by intramammary infusion of *S. aureus* into all quarters. Infection status was then determined 2, 3, and 4 wk PI. The range of resulting infection status included 20.7%

of cows that did not establish infection in any of the quarters, 15.6% of cows with 1 quarter infected, 25.9% with 2 quarters infected, 17.8% with 3 quarters infected, and 20.0% with all quarters infected. Thus, it seems clear from their study that some cows are inherently more resistant to *S. aureus* mastitis than other cows. Our hypothesis was that a range of endogenous innate responsiveness to mammary gland infection exists within the cow population and that the high-responsive phenotype is the one more prone to develop severe mastitis associated with greater tissue damage and compromised return to full production (Kandasamy et al., 2011). Our goal was to develop an ex vivo technique that could be of use in identifying genetic polymorphisms or epigenetic effects contributing to an animal's innate immune response phenotype, leading to more accurate breeding and selection strategies aimed at reducing mastitis severity. Thus, determination of an animal's innate response phenotype at a young age (Green et al., 2011) could be of use in selecting those calves that would be ideal candidates for milking herd replacements as opposed to being more appropriate for beef production. Current costs of raising herd replacements are estimated at greater than $1,500, depending on farm-management practices (Heinrichs et al., 2013). Testing of existing animals at a young age would also incorporate the effects of epigenetic modification on gene expression that may have occurred from early life environmental conditions.

Use of primary fibroblasts as an ex vivo model system is well documented for other species (Rogers et al., 2007; Wang et al., 2011; Schramm et al., 2012), demonstrating the ability of these cells to produce inflammatory markers, such as IL-6 and IL-8, or exhibit altered gene expression in response to various stimuli, such as LPS or Pam2CSK4. Kandasamy et al. (2011) used dermal fibroblasts as a model cell to predict

the immune responses of dairy cattle following an intramammary *E. coli* challenge. In their study, animals classified as a high-responding phenotype experienced more mammary tissue damage, and an elevated SCC during the resolution phase of the infection compared with the low-responding animals, yet rates of bacterial clearance were similar between phenotypes. In the current study, we used a similar strategy to examine the relation between fibroblast stimulation with Pam2CSK4 and host immune responses following a *S. aureus* challenge.

Previously, we found inconsistent and generally modest production of IL-8 by fibroblast cultures when stimulated with live, heat-killed, or UV-killed *S. aureus* (data not presented). Others have also reported limited responses of cultured mammary epithelial cells to heat-inactivated *S. aureus* (Günther et al., 2011; Fu et al., 2013). As a substitute, we choose to use stimulation with the synthetic ligand, PAM2, which has been shown to activate the TLR2/6 pathway in murine macrophages and B lymphocytes in a similar manner as gram-positive bacteria (Buwitt-Beckmann et al., 2005). Our fibroblasts secreted reproducible and easily detectable levels of IL-8 following PAM2 treatment, with a range in responsiveness among the cultures from the 50 individual cows. Furthermore, we found significant correlations between 2 proinflammatory markers (IL-8 and IL-6) that are produced following activation of the TLR2/6 pathway, indicating that the range in IL-8 response phenotype of these animals was not solely due to polymorphisms in the IL-8 gene. We further went on to demonstrate the stability of the IL-8 response phenotype over time in cultures established from replicate biopsies obtained before and after the animals were challenged with *S. aureus*, and found that the response phenotype was not altered after the experimental intramammary infection.

Finally, the response to PAM2 was mirrored to some extent by the responses to LPS and IL-1β. These 2 ligands are recognized via different receptors (TLR4 and the IL-1β receptor, respectively); however, upon ligand recognition, portions of the intracellular signaling cascades from all 3 ligands converge, leading to common activation of downstream transcription factors such as NF-κB, activator protein 1 (AP1), and interferon regulatory factor 3 (IRF3) (Flannery and Bowie, 2010; Takeuchi and Akira, 2010). This moderate similarity between responses to various ligands suggests that fibroblast response phenotype to PAM2 is not solely due to ligand recognition, but also incorporates signaling pathway differences.

We selected 5 low- and 5 high-ranked animals to determine if their ex vivo phenotype was reflected in their in vivo response to an intramammary challenge with *S. aureus*. The selected challenge strain was originally recovered from the mammary glands of several animals at a Vermont dairy farm that were experiencing chronic, subclinical infections with elevated milk SCC (Barlow et al., 2013). It was determined to be of the sequence type 8 multilocus sequence type that has also been isolated from other naturally infected dairy animals in Turkey (Türkyilmaz et al., 2010), The Netherlands (van den Borne et al., 2010), and Switzerland (Sakwinska et al., 2011).

Whereas milk SCC increased in both the HR and LR groups following the challenge, a more rapid influx of immune cells was observed in the HR. Considerable increases of IL-8 in milk from HR following the challenge likely played a role in the greater influx of neutrophils into the infected gland due to its role as a chemoattractant. However, this chemokine is but one representative member of a family of neutrophil chemoattractants, such as chemokine (C-X-C motif) ligand (CXCL)1, CXCL2, CXCL3,

and CXCL6, which are induced in bovine cells following stimulation of the TLR pathway; as such, IL-8 may not be the only chemokine contributing to differences in neutrophil influx (Griesbeck-Zilch et al., 2009; Kandasamy and Kerr, 2012). The more rapid and sustained influx of neutrophils in the HR offered little advantage in containing the infection. Rather, 2 of the 5 LR were able to initially clear the infection; although 1 was reinfected 13 d later or perhaps had not completely cleared the infection. In addition, the LR animals suffered less severe mammary damage when using milk BSA as an indicator of breakdown of the mammary epithelial barrier.

A considerable body of evidence exists suggesting that physiological states such as early lactation result in immunosuppression leading to a reduced functionality of neutrophils entering into the infected gland (Burvenich et al., 2007). It has also been suggested in several review articles that a rapid influx of cells into the gland is a major factor in controlling *E. coli* mastitis (Paape et al., 2002; De Schepper et al., 2008). However, this suggestion is not easily tested. In one study, 19 early lactation cows were classified as being moderate or severe responders based on lower or higher reactive oxygen species-generating capacity of their blood neutrophils. All animals were then challenged with intramammary *E. coli* and the severe responders had a slightly delayed increase in SCC compared with the moderate responders, as well as an increase in milk colony-forming units (Vandeputte-Van Messom et al., 1993). Further studies are needed to evaluate this delayed response hypothesis. Studies with immunosuppressive agents that cause a delay in neutrophil influx could be of use in evaluating the role of moderate changes in the speed of neutrophil influx in containing mastitis pathogens. Interestingly, in cows chronically infected with *S. aureus*, a 3-d treatment with cortisol had little effect

on milk SCC or colony-forming units, whereas the more potent dexamethasone had marked effects on reducing milk yield, which were reflected in large increases as opposed to immunosuppressive decreases in both milk SCC and colony-forming units (Burton and Kehrli, 1995).

In contrast to delayed influx, several cytokine-based strategies have been investigated as a means to stimulate additional neutrophil recruitment. These studies have revealed inconsistent effects as mastitis therapeutics (Sordillo et al., 1997). Recently, Kauf et al. (2007) investigated the use of LPS as a possible treatment for *S. aureus* mastitis due to its immunostimulating properties with the hypothesis that a greater inflammatory response can enhance clearance of the bacteria. They infected mammary quarters with *S. aureus* and then 24 h later LPS or saline control was infused into challenged glands. The LPS treatment rapidly caused large increases in SCC and milk BSA and a moderate reduction in colony-forming units at the peak of the acute response. However, bacteria recovery rates from these quarters were actually higher for the remainder of the induced inflammation period. The results clearly indicated that, at least with a *S. aureus* mastitis, additional recruitment of leukocytes into the gland did not aid in bacterial clearance and may actually be detrimental by causing further damage to the epithelial layer, thus exposing additional sites for bacterial attachment. In our study, the heightened inflammatory response and over production of immune mediators in the HR animals led to higher amounts of collateral damage in the mammary gland, whereas the delayed and reduced influx of neutrophils observed in the LR was associated with a general trend of reduced bacterial burden.

It has been shown that anti-inflammatory treatments administered around the time of an intramammary challenge can reduce the severity of clinical symptoms and promote restoration of prechallenge milk production and quality (Lohuis et al., 1988; Yeiser et al., 2012; Sipka et al., 2013). Similarly, the use of pharmaceutical agents aimed at reducing the magnitude of the immune response has demonstrated positive effects in human health. For instance, the pathologies of several human diseases, including rheumatoid arthritis (McInnes and Schett, 2007), psoriasis (Tamilselvi et al., 2013), and type 2 diabetes (Crook, 2004), are due to an excessive immune response with large numbers of infiltrating immune cells and abnormally high production of proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α. Many of the treatment options for these diseases focus on reducing or inhibiting immune mediators, such as tumor necrosis factor-α (Armuzzi et al., 2014), the JAK (Janus tyrosine kinase) pathway (Hsu and Armstrong, 2014), and IL-1β (Dinarello, 2011). Use of anti-inflammatories as a treatment option in bovine mastitis requires further investigation to determine what magnitude of immune response is effective for clearing a mammary infection while minimizing the damage to the host, as well as the effect of variable immune responses in the success or failure of mastitis treatments.

The development of a vaccine that generates an effective antibody response against *S. aureus* has been the focus of many studies (Leitner et al., 2003; Lee et al., 2005; Middleton et al., 2006). In each of these studies, the levels of sera antibodies toward *S. aureus* increased following vaccination. To determine the level of protection conferred from the vaccine, experimental intramammary challenges were completed in 2 of these studies. Leitner et al. (2003) administered a vaccine containing 3 *S. aureus*

strains causing mastitis in dairy cows and then subsequently challenged vaccinated animals with 1 of those strains. Animals receiving the vaccine were protected against an IMI significantly more than controls, with infection rates of 35 and 90% for vaccinated and control animals, respectively. Clinical symptoms, including SCC, were milder in the vaccinated animals post challenge as well. In the second study, Middleton et al. (2006) performed an intramammary challenge on 47 animals, 36 of which had been vaccinated against *S. aureus*. Rates of persistent *S. aureus* mammary infections were similar between the vaccinated and control animals and no differences in milk SCC, bacteria counts, or clinical mastitis scores were observed. The conflicting results between vaccine effectiveness could be due to different vaccine formulations between studies, but it also reinforces the fact that variation is present in the immune response between animals, even following vaccination.

Within our challenged animals, activation of the adaptive immune system was confirmed by an increase in total IgG antibody levels to *S. aureus* from pre challenge levels in both milk and plasma in the HR animals, although the LR animals showed a much smaller increase. To further examine the antibody-mediated response, we chose to focus on the 2 predominant IgG isotypes of the mammary gland, IgG1 and IgG2, and how their concentrations in the milk would be influenced after an experimental mammary infection. The IgG1 isotype is the predominant one found in mammary secretions of both healthy and infected glands, primarily due to its selective transport across the epithelial lining by the IgG Fc receptor (Barrington et al., 1997). Concentrations of IgG2, normally low in healthy milk, will increase quickly after mammary infections due to the antibody binding to the Fc receptor on neutrophils in the blood and being co-recruited to the site of

infection (Atalla et al., 2010). In agreement with other experimental challenge models (Bourry and Poutrel, 1996; Tollersrud et al., 2006), there was an increase in anti-*S. aureus* total IgG present in whey and plasma, and both IgG1 and IgG2 observed in the whey from the HR animals. However, elevated concentrations of strain-specific antibodies did not offer an advantage in pathogen clearance for the HR animals. In the current study, basal levels of serum and milk antibodies were similar between the 2 groups. It is unknown if higher levels of preexisting antibodies would have mitigated the propensity for an animal to develop a chronic infection following an experimental challenge.

A panel of genes was selected to further investigate the basis of the low- versus high-response phenotype of the fibroblasts from the 10 challenged animals. Differences in expression levels of receptors and proteins involved in pathogen recognition can affect the propensity for an animal to develop an infection (Fonseca et al., 2011; Kandasamy and Kerr, 2012; Novak, 2014). Enhanced pathogen detection, due to genetic or epigenetic differences between animals, could be a major factor differentiating the high versus low phenotype observed in our fibroblasts. Koets et al. (2010) observed an SNP in the extracellular portion of the bovine TLR2 gene that was more prevalent in animals seemingly resistant to *Mycobacterium avium paratuberculosis* infection. This SNP resulted in greater proinflammatory cytokine production from monocytes as well as enhanced T-cell activation following challenge. The extracellular receptors (TLR2 and TLR6) are thought to play a key role in detection of *S. aureus* (Nakayama et al., 2012) and we observed greater expression of both receptors in high-responding cultures following PAM2 stimulation. This greater expression would correspond to the

significantly enhanced transcription of IL-6, and clear trend for enhanced transcription of CCL20 and TNF-α.

Tumor necrosis factor-α and IL-6 are considered potent proinflammatory cytokines that can activate lymphocytes and the production of acute phase proteins (Bannerman, 2009). Elevated concentrations of serum IL-6 have been shown to be correlated with disease severity in naturally infected cows (Hagiwara et al., 2001), suggesting that IL-6 can be used as a second marker for a high-responding phenotype. Expression levels and protein concentrations of IL-6 were greater in our HR fibroblast cultures, perhaps reflecting the enhanced TLR2 and TLR6 expression in these cells compared with LR cultures. Quite surprisingly, we did not find a significant difference in IL-8 gene expression between the groups after stimulation with PAM2. The slight numerical difference in gene expression at 2 and 8 h post-PAM2 as measured by qRT-PCR appears insufficient to cause the nearly 4-fold difference in protein accumulation in media 24 h post-PAM2. This suggests that determination of actual protein production at 24 h post challenge is a more sensitive indicator of the response phenotype. In a similar previous study (Kandasamy and Kerr, 2012) we did find 3-fold higher IL-8 gene expression following an 8-h stimulation of selected high- versus low-responding fibroblast cultures with LPS. Ideally, the response phenotype would be best classified using a combination of the production of multiple proteins and the expression of a panel of inflammation associated genes.

In the present study, we presented evidence for the use of dermal fibroblasts as a cell model for a dairy animal's in vivo immune response following an intramammary challenge with *S. aureus*. Animals selected as HR based upon their cellular response

mounted a stronger innate immune response demonstrated by higher levels of IL-8 and SCC present in the milk of the challenged gland compared with LR. Tissue damage to the infected quarter, determined by increased levels of BSA in whey, was more extensive in the HR group as well. High-responding animals also elicited a stronger adaptive immune response, indicated by an increase in anti-*S. aureus* antibodies in whey from the challenge quarter, but no protective advantage was observed for these animals. The LR and HR animals were initially chosen based on extreme differences in the responsiveness of their fibroblasts following challenge with TLR agonists, and these differences were corroborated in the immune responses following an intramammary challenge. Our study indicates that dermal fibroblasts may be useful to explore potential genetic or epigenetic causes for the diversity in immune responses to mastitis causing pathogens between dairy animals.

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Table 1 Oligonucleotide primers used for real-time PCR quantification.

(The top and bottom sequences are the forward and reverse, respectively.)

Gene Name	Sequence (5' to 3')	Reference
TLR2	GGTTTTAAGGCAGAATCGTTTG AAGGCACTGGGTTAAACTGTGT	Akira, Takeda, and Kaisho (2001)
TLR6	CCTTGTTTTTCACCCAAATAGC TAAGGTTGGTCCTCCAGTGAGT	Akira, Takeda, and Kaisho (2001)
IL-8	GCTGGCTGTTGCTCTCTTG AGGTGTGGAATGTGTTTTTATGC	Pareek et al. (2005)
CCL20	TTCGACTGCTGTCTCCGATA GCACAACTTGTTTCACCCACT	Gilbert et al. (2013)
IL-6	TGAGGGAAATCAGGAAAATGT CAGTGTTTGTGGCTGGAGTG	Pareek et al. (2005)
TNF-α	TCTTCTCAAGCCTCAAGTAA CCATGAGGGCATTGGCATAC	Bougarn et al. (2011)
B- actin	GCAAATGCTTCTAGGCGGACT CAATCTCATCTCGTTTTCTGCG	Pareek et al. (2005)

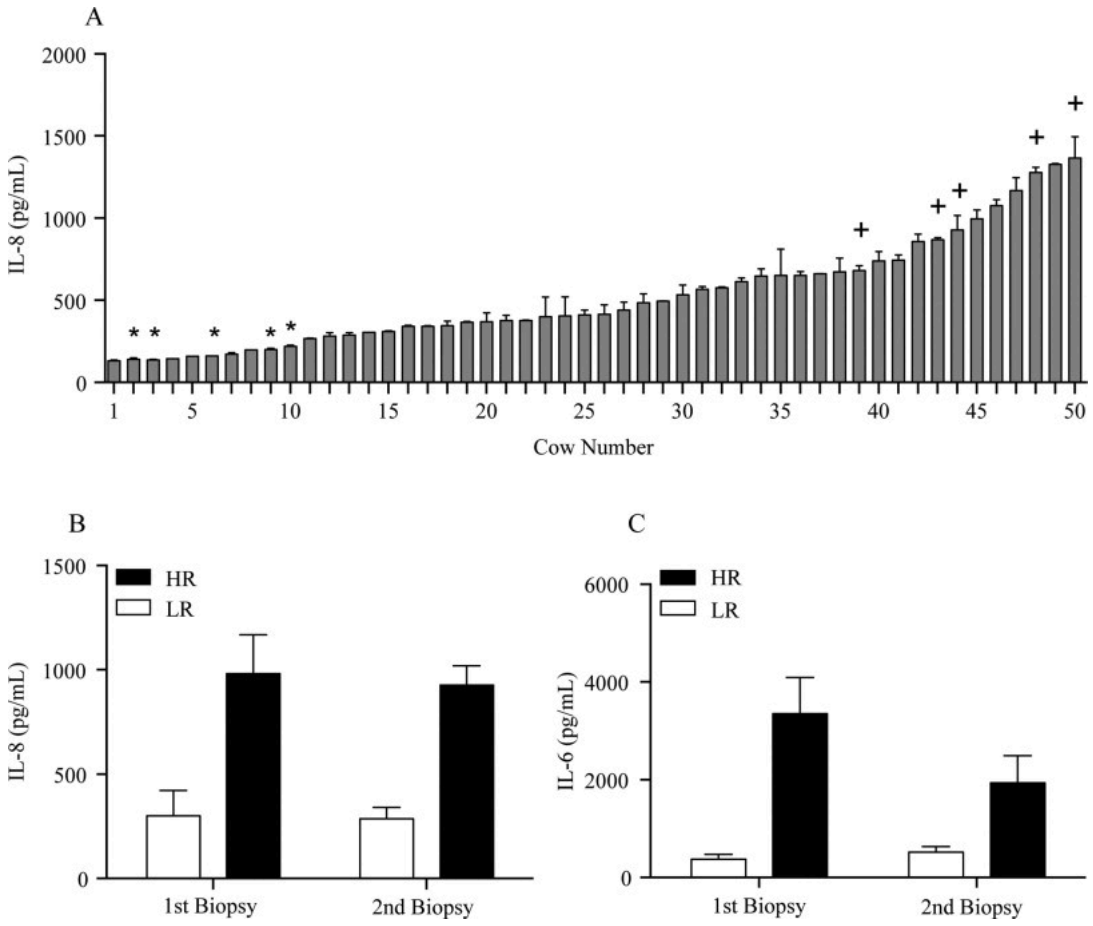

Figure 1 Production of IL-8 and IL-6 by cultured dermal fibroblasts.

(A) Concentrations of IL-8 in fibroblast culture media following a 24 h challenge with 200 ng/ml of Pam2SCK4 (Pam2). An * and a + indicate the low responding (LR) and high responding (HR) animals, respectively, that were selected for an in vivo *S. aureus* challenge. Cells cultured in media alone (negative control) produced no detectable IL-8. Values are mean ± SEM of two replicates. (B) IL-8 and (C) IL-6 protein concentrations in media following a 24 h challenge with Pam2 of fibroblasts isolated from the selected animals approximately five months before, and six weeks after an experimental challenge with *S. aureus*. For the first biopsy, n=5 LR and n=5 HR, for the second biopsy n=5 LR and n=4 HR (one 2nd HR biopsy did not yield a viable culture due to microbial contamination and was discarded). Concentrations of IL-8 and IL-6 were similar between replicated biopsies from animals within the LR and HR groups ($P > 0.05$; paired t-test). Media wells had no detectable IL-8 or IL-6.

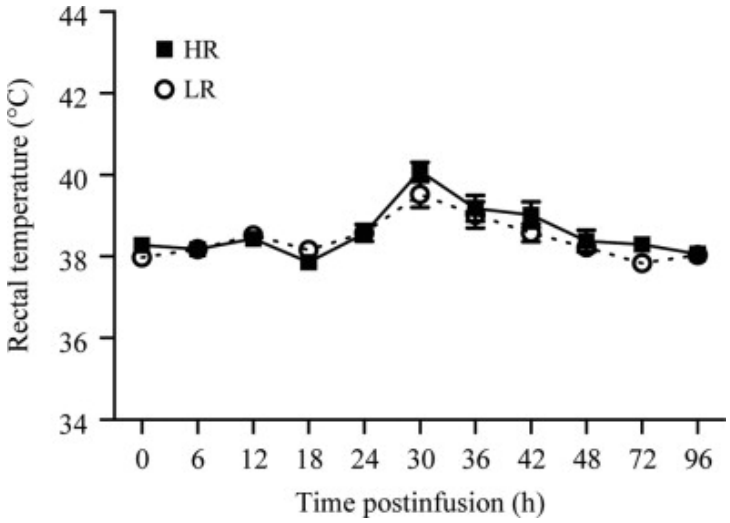

Figure 2 Rectal temperatures post challenge.

Rectal temperatures were monitored after a single intra-mammary infusion of *S. aureus* in the right hind quarter of ten challenged animals. Values are expressed as mean ± SEM. There were no significant differences in rectal temperatures following the challenge between low responder (LR) and high responder (HR) animals.

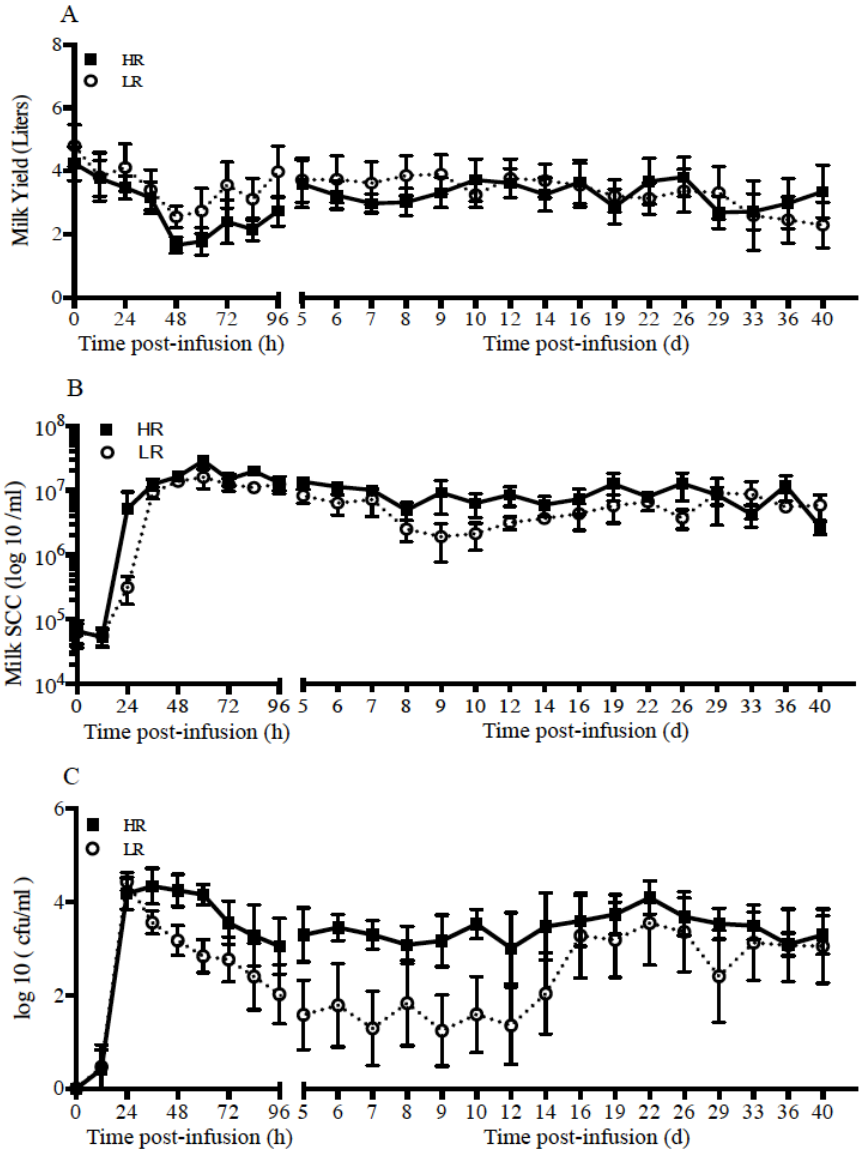

Figure 3 Challenged quarter milk yield, milk SCC, and *S. aureus* cfu following intra-mammary challenge.

The LR animals (n=5) and HR animals (n=5) received an infusion of *S. aureus* in the right hind quarter, after which, milk yield from the infected quarter (A), milk SCC (B), and bacteria counts (C) were monitored. Milk SCC and bacteria counts were $\log_{10}$ transformed. Values are expressed as mean ± SEM. There was no significant difference in milk yield between LR and HR animals during the challenge. Milk SCC was different between the groups throughout the challenge, with low responding animals experiencing reduced SCC ($P < 0.05$) SCC in milk from the right hind quarter when compared to the high responders. *S. aureus* was recovered from the right hind quarter of all animals by 24 h post-challenge. There was no significant difference in average milk bacteria count between the low and high responding animals throughout the trial, although two of the

low responders cleared the infection (days 3 and 4), with one of these becoming measurably reinfected by day 14. The other three quarters of each cow served as control quarters and were culture negative throughout the challenge.

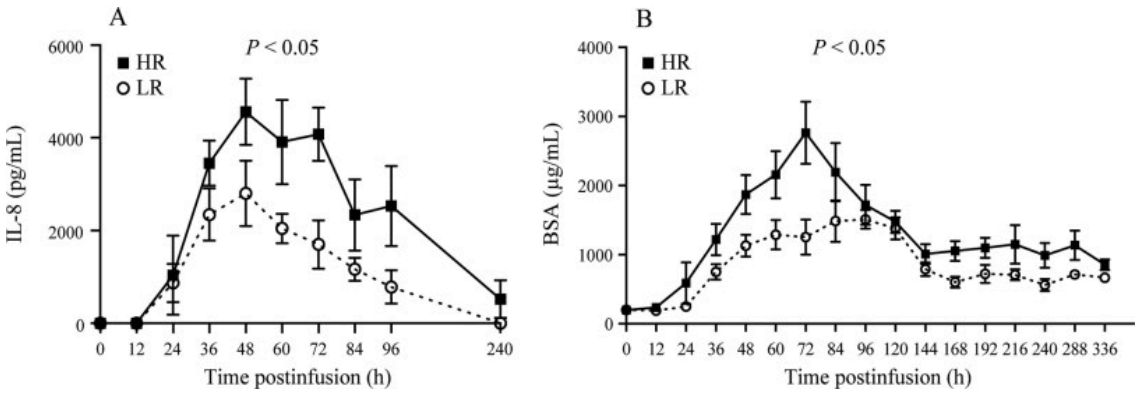

Figure 4 Levels of inflammatory markers in challenge quarter whey.

Concentrations of IL-8 (A) and bovine serum albumin (B) were determined in whey from the right hind quarter of the LR animals (n=5) and HR animals (n=5) following an intra-mammary infusion of *S. aureus*. Values represent the mean ± SEM for the two groups. The high responders had increased ($P < 0.05$) IL-8 and BSA concentrations when compared to the low responders.

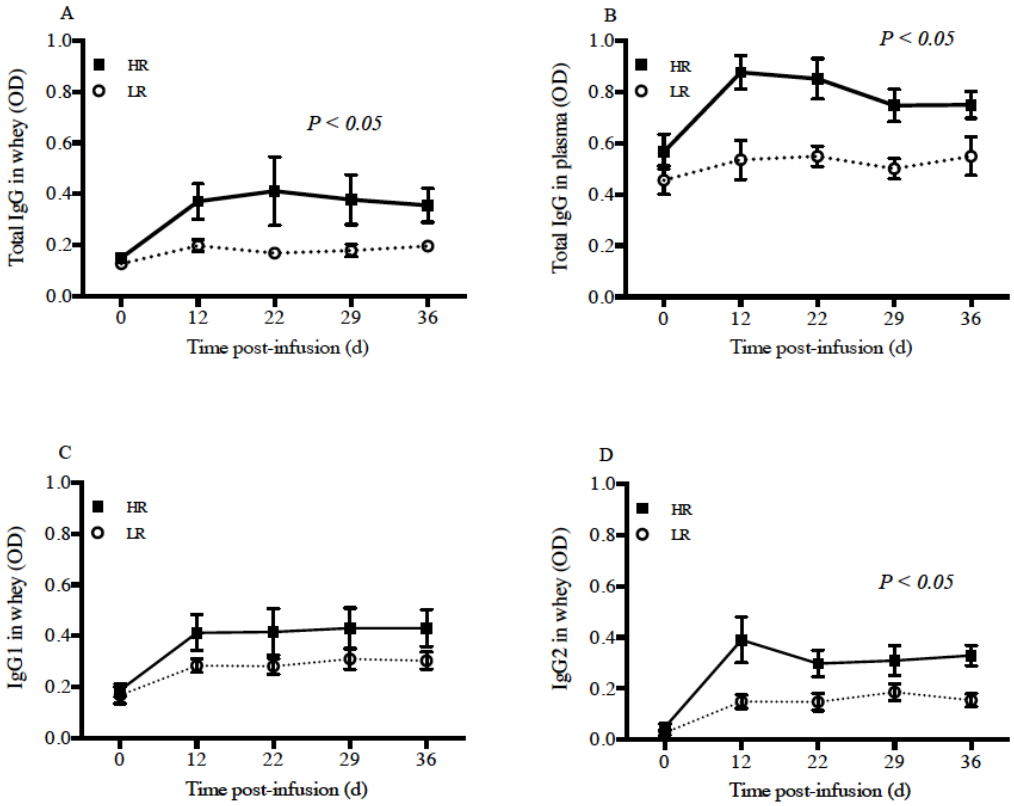

Figure 5 Anti-*S. aureus* antibodies present in blood plasma and whey from the right hind quarter.

Total levels of anti-*S. aureus* IgG were determined via an ELISA (represented as OD values) in whey and plasma of the five LR and five HR animals following challenge. Infection by *S. aureus* increased levels of total IgG in challenge quarter whey (A) and plasma (B) in both groups, and they were significantly greater ($P < 0.05$) in the HR compared to the LR animals. The levels of two IgG isotypes, IgG_1 and IgG_2, in whey from the challenged quarter were determined as well, and are presented in (C) and (D), respectively. Values represent the mean ± SEM of the LR and HR animals. The HR animals had an increase ($P < 0.05$) in total levels of IgG_2 in whey compared to the LR animals, while IgG_1 levels were similar between LR and HR animals.

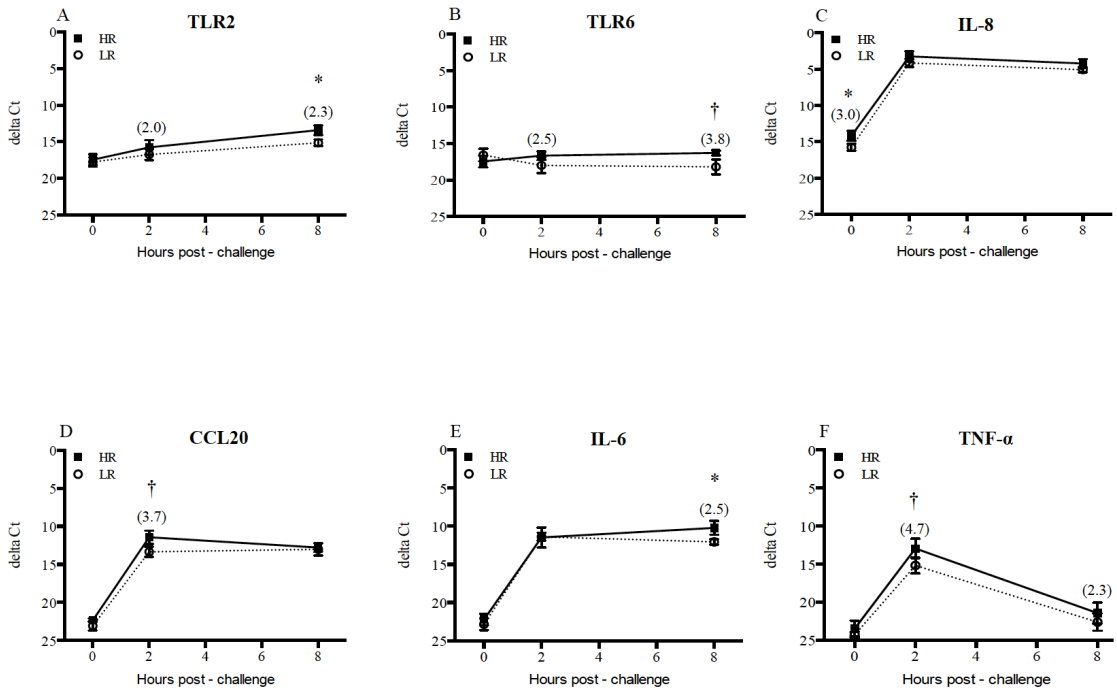

Figure 6 Real-time PCR quantification of messenger RNA.

Aliquots of dermal fibroblasts from the ten challenged animals (n=5 LR, n=5 HR) were revived, treated with Pam2 (200 ng/ml), and total RNA was extracted at hour zero (control conditions), two and eight for quantification of gene expression. Results are expressed as delta Ct in comparison to β-actin. Expression fold differences between LR and HR cultures are indicated by the values in parenthesis above 0, 2, or 8 h time points, with values indicating the greater expression in HR cultures. † indicates $P < 0.1$, * indicates $P < 0.05$. Values are mean ± SEM.

Chapter 3: Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome

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3.1 Abstract

Background: We have previously found substantial animal-to-animal and age-dependent variation in the response of Holstein fibroblast cultures challenged with LPS. To expand on this finding, fibroblast cultures were established from dairy (Holstein) and beef (Angus) cattle and challenged with LPS to examine breed-dependent differences in the innate immune response. Global gene expression was measured by RNA-Seq, while an epigenetic basis for expression differences was examined by methylated CpG island recovery assay sequencing (MIRA-Seq) analysis.

Results: The Holstein breed displayed a more robust response to LPS than the Angus breed based on RNA-Seq analysis of cultures challenged with LPS for 0, 2, and 8 hours. Several immune-associated genes were expressed at greater levels (FDR < 0.05) in Holstein cultures including *TLR4* at all time points and a number of pro-inflammatory genes such as *IL8, CCL20, CCL5*, and *TNF* following LPS exposure. Despite extensive breed differences in the transcriptome, MIRA-Seq unveiled relatively similar patterns of genome-wide DNA methylation between breeds, with an overall hypomethylation of gene promoters. However, by examining the genome in 3Kb windows, 49 regions of differential methylation were discovered between Holstein and Angus fibroblasts, and two of these regions fell within the promoter region (-2500 to +500 bp of the transcription start site) of the genes *NTRK2* and *ADAMTS5*.

Conclusions: Fibroblasts isolated from Holstein cattle display a more robust response to LPS in comparison to cultures from Angus cattle. Different selection strategies and

management practices exist between these two breeds that likely give rise to genetic and epigenetic factors contributing to the different immune response phenotypes.

Keywords: Innate immunity, DNA methylation, RNA-Seq, MIRA-Seq

3.2 Background

The innate immune response plays a critical role in pathogen detection and the resulting inflammatory response that arises to contain and eliminate foreign invasions into host tissues. Infection of the mammary gland resulting in mastitis is an important and well characterized disease of dairy cattle, such as the Holstein breed (Bannerman, 2009). However, the incidence and severity of this disease in beef cattle has received little attention although two recent studies that examined mastitis prevalence in beef cows reported a moderate incidence with a surprising lack of *Escherichia coli* induced mastitis (Paape et al., 2000, Persson Waller et al., 2014). This pathogen is a major cause of mastitis in dairy animals and thus differences in susceptibility to *E. coli* infection may exist between dairy and beef breeds. Bovine mammary infections due to *E. coli* typically cause an acute response with inflammation ranging from mild and quickly resolving, to severe forms that can lead to sepsis, shock, and even death (Schukken et al., 2011). The response is initiated following recognition of *E. coli* lipopolysaccharide (LPS) by the extracellular Toll-like receptor 4 (TLR4), which is expressed on multiple cell types within the mammary gland (Gilbert et al., 2013).

While there are multiple factors that can influence the inflammatory response to mammary gland infection, it has been suggested that host factors play the largest role in determining the severity of *E. coli* mastitis (Burvenich et al., 2003). These host factors

include age, stage of lactation, vaccination history, as well as genetic, and possibly epigenetic mechanisms all contributing to the magnitude and resolution of the host response. Identification of genetic or epigenetic factors controlling the inflammatory response may lead to enhanced selection or management strategies to produce dairy animals that have reduced collateral damage from mastitis. For example, genetic polymorphisms within pathogen recognition receptors or signaling pathway intermediates have been shown to lead to differences in disease susceptibility (von Bernuth et al., 2008, Kataria et al., 2011). Epigenetic mechanisms, such as DNA methylation or histone modifications can also influence gene expression resulting in phenotypic variation in immune response (Bayarsaihan, 2011). For example, human intestinal epithelial cells have increased DNA methylation in the *TLR4* gene promoter that reduces its expression and leads to a reduced responsiveness to LPS (Takahashi et al., 2009).

Selective breeding of cattle has been employed for many generations to produce breeds differing in economically important traits such as enhanced milk production in dairy breeds, and greater feed efficiency and meat quality in beef breeds (Miglior et al., 2005, Kause et al., 2015). Cattle breeding has even resulted in sub-species (*Bos taurus* and *Bos indicus*) that differ markedly in resistance to disease such as that caused by tick infestation (Piper et al., 2008, Piper et al., 2010) or challenge with *Theileria annulata* (Glass et al., 2005a). Results from these studies suggest that *B. taurus* animals develop a greater inflammatory response to pathogens than do breeds of the *B. indicus* subspecies. Within the *B. taurus* sub-breed, studies directly comparing the inflammatory response to mammary infection between dairy and beef breeds have not been conducted. To further investigate potential differences in immune responses between dairy and beef animals,

we challenged dermal fibroblasts obtained from Holstein (dairy) and Angus (beef) cows with LPS and then used RNA-Seq to examine global gene expression differences. We also employed MIRA-Seq to examine if global differences in the methylome could explain differences in the transcriptome.

3.3 Results

3.3.1 Fibroblast IL-8 Response

Dermal fibroblasts (DF) isolated from Holstein and Angus heifers were used to investigate differences in the innate immune response between dairy and beef breeds. Fibroblasts isolated from Holsteins produced considerably higher levels of IL8 ($P < 0.01$) in response to a 24 h LPS (100ng/ml) challenge (Fig. 1a) when compared to Angus cultures (703 ± 86 pg/ml vs. 322 ± 46 pg/ml, respectively). A similar pattern was observed following IL1B (1ng/ml) treatment (Fig. 1a), with Holstein DF having a greater magnitude of IL8 response than Angus DF (1355 ± 160 pg/ml vs. 639 ± 80 pg/ml, respectively). There was no detectable IL8 in media collected from un-stimulated Holstein or Angus fibroblasts.

RNA-Seq and MIRA- Seq analysis was performed on eight cultures ($n = 4$/breed) randomly selected from the pool of animals. These cultures were revived simultaneously from cryopreservation and prepared for a second challenge with LPS and IL1B. The Holstein cultures produced approximately twice ($P < 0.01$) as much IL8 protein in response to LPS compared to the Angus cultures (Fig. 1b). Similarly, differential IL8

responses were observed following IL1B exposure, with Holstein cultures exhibiting a hyper-responsive phenotype in IL8 protein production (Fig. 1b).

3.3.2 RNA-Seq analysis of Holstein and Angus LPS response

Total RNA samples for RNA-Seq analysis were obtained from the four Holstein and four Angus cultures following LPS exposure for 0, 2, and 8 h. These 24 samples generated approximately 54 million reads per sample following quality control. Alignment to the UMD v3.1 bovine genome resulted in 95% of the reads falling within alignment parameters (see Methods), thus an average of 51 million reads per sample. Under our definition of expression (CPM > 1 in at least 50% of the samples), there were 20,356 targets detected at hour 0, which defined the core bovine fibroblast transcriptome under basal conditions. At hours 2 and 8 following LPS treatment, there were 21,411 and 21,590 targets detected, respectively. By combining breeds, and comparing basal gene expression to time points following LPS-induced innate immune response, 624 transcripts were revealed to have differential gene expression (FDR < 0.05; CPM > 1; FC ≥ 2) with 470 up- and 154 down-regulated at hour 2 post-LPS (Additional file 1). At hour 8 post-LPS, 331 transcripts were discovered having differential gene expression compared to hour 0, of which, 250 were up- and 81 were down- regulated (Additional file 2). LPS treatment of the fibroblast cultures induced various innate immune-associated genes in the LPS signaling pathway and those coding for response proteins (Table 1). There were temporal differences in the induction of these genes following LPS exposure for 2 or 8 hours. Early induced genes included several pro-inflammatory chemokines and cytokines such *IL8, IL1A, TNF, chemokine (C-X-C) motif ligand* (*CXCL*) *2,* and

Interferon regulatory factors (*IRF*) *1* and *5*. Following 8 h of LPS exposure, several Type I IFN-related genes showed greater expression compared to hour 0, while *TNF* expression had returned to basal levels.

A comparison between Holstein and Angus cultures revealed 844, 968, and 730 differentially expressed (DE) genes (FDR < 0.05; CPM > 1; FC ≥ 2) at hours 0, 2, and 8-post LPS, respectively (Fig. 2; Additional file 3). Of these genes, 369, 517, and 477, respectively, had higher expression levels in Holstein cultures compared to Angus cultures. Pathway enrichment analysis was completed with DAVID and genes with differential expression between the breeds at 2 h post-LPS were involved with the MAPK (P = 0.029) and TGF-beta (P = 0.031) signaling pathways. By 8 h post-LPS, the NOD-like (P = 0.003), Toll-like (P = 0.02), and RIG-I-like (P = 0.02) receptor signaling pathways were represented in our set of DE genes between the two breeds. Table 2 lists several of the DE genes that are involved in the innate immune response. *TLR4*, *NOD1*, and *TRAF1*, genes involved with the recognition of pathogen associated molecular patterns and activation of NF-κB pathway, were expressed higher in Holstein cultures. Chemokines such as *chemokine (C-C motif) ligand (CCL) 2*, *5*, and *20*, and *IL8* that recruit monocytes, T cells, lymphocytes, and neutrophils to sites of infection also had higher expression levels in Holstein fibroblasts. In addition to genes related to the innate immune response, differences were observed in genes that have been implicated in DNA methylation, such as the family of DNA methyltransferase (DNMT) genes and *ubiquitin-like with PHD and RING finger domains 1* (*UHRF1*) (Bostick et al., 2007, Moore et al., 2013). Expression of *DNMT3A* and *3B*, which are involved with *de novo* DNA methylation, were similar between the breeds. However, expression of *DNMT1*, which is

responsible for maintenance of DNA methylation patterns following DNA replication, was 1.6-fold higher at hour 0 and 1.5-fold higher at hours 2 and 8 in Angus cultures compared to Holstein cultures. Angus cultures also had 2.3-, 2.0-, and 2.0-fold higher expression of *UHRF1* at 0, 2, and 8 hours post-LPS treatment (Table 3). Interestingly, greater activation of a potentially anti-inflammatory signaling pathway in Angus cultures was noted by the 3.1-fold higher expression of the gene encoding *chemokine (C-X-C motif) receptor 4 (CXCR4)* at hour 0 compared to Holsteins. While *CXCR4* expression was not different at hours 2 and 8 post-LPS, the sole ligand of this receptor, *CXCL12*, was expressed 5.7-, 7.4-, and 6.5-fold higher in Angus cultures at hour 0 and at 2 and 8 hours post LPS stimulation.

3.3.3 RT-qPCR Confirmation of RNA-Seq

Several immune-response associated genes were selected for RT-qPCR confirmation of expression differences observed between Holstein and Angus cultures following LPS treatment as determined by RNA-Seq. *TLR4*, *IL8*, *CCL20*, *CCL5*, and *TNF* showed similar expression levels in RT-qPCR analysis in comparison to the RNA-Seq data set. Basal expression of *IL8*, *CCL20*, *CCL5*, and *TNF* was similar between the breeds. However, by 2 h after LPS stimulation, marked differences were observed between Holstein and Angus expression of *IL8*, *TNF*, *CCL20*, and *CCL5*, with a 3.0-, 24.3-, 4.9-, and 3.9- fold higher expression in Holstein fibroblasts compared to Angus fibroblasts (Fig. 3 b, c, d, and e). A similar pattern was observed at 8 hours, with Holstein cultures having higher expression in the following genes: *IL8* (13.1-fold), *TNF* (13.0-fold), *CCL20* (35.3-fold), and *CCL5* (17.8-fold). LPS exposure did not induce *TLR4*

expression in either breed; however, Holstein cultures consistently expressed higher levels of *TLR4* compared to Angus cultures (Fig. 3a; 4.5-, 5.7-, and 3.1- fold higher at hours 0, 2, and 8, respectively). In general, gene expression values determined by RT-qPCR are in agreement with the transcriptomic results from RNA-Seq data.

3.3.4 MIRA-Seq

The 8 DNA samples (4 per breed) processed for MIRA-Seq analysis resulted in approximately 44 million reads per sample following quality control. Alignment to the bovine UMD v3.1 reference genome yielded 97% of reads falling within alignment parameters (see Methods) and an average of 43 million mapped reads per sample. To determine differential methylation rates between Holstein and Angus fibroblasts, the genome was divided into 3Kb windows, and then the read count in each window was compared. Analysis of the 3Kb windows revealed 49 regions that had different levels of methylation between Holstein and Angus cultures based on the following thresholds: FDR < 0.1; CPM > 1; FC ≥ 2 (Table 3). Of these differentially methylated regions (DMR), 24 had higher rates of methylation in cultures from Holstein animals, while 25 displayed higher methylation levels in Angus fibroblasts. Of the 24 regions with higher methylation in Holstein, 14 were found partially within an annotated gene while 10 were not. Within the 25 DMR with higher methylation levels in Angus cultures 13 fell within annotated genes while the remaining 12 regions did not. Only two of the DMR (greater methylation in Angus cultures) were located within the defined promoter regions of genes: *a disintegrin and metalloprotease with thrombospondin motifs 5* (*ADAMTS5*) and *neurotrophic tyrosine kinase, receptor type 2* (*NTRK2*). These DMRs were not reflected

by differences in expression of either gene between the Holstein and Angus cultures at hours 0, 2, and 8 post-LPS (Table 3). A DMR spanning intron 3 and exon 4 of the *agouti signaling protein* (*ASIP*) gene had greater methylation in the Angus cells and was associated with a marked increase in its expression in these cells at all time points.

3.3.5 Influence of DNA methylation on gene expression

The genome-wide association between gene expression and methylation levels within a gene body or gene promoter region was investigated by plotting MIRA-Seq values for a gene body or gene promoter against RNA-Seq values for 8 h post-LPS. As data from the two breeds appeared similar, the combined data points from both the Holstein and Angus cultures were binned as either low or high, with a gating value of reads per kilobase per million matched reads (RPKM) =5 for RNA-Seq or RPKM = 0.5 for MIRA-Seq. When the relationship between methylation levels in either the gene promoter region or the gene body and that gene's subsequent expression level was analyzed, it revealed the two values were significantly dependent upon one another ($P < 0.001$; O.R. = 0.83; 95% C.I. = 0.76 – 0.89 and $P < 0.001$; O.R. = 1.26; 95% C.I. = 1.14 – 1.40), for gene promoter (Fig. 4a) and gene body (Fig. 4b), respectively. This indicates a strong inverse relationship between methylation in either the gene promoter or gene body and the level of gene expression. Analysis of the 0- and 2-hour time points following LPS treatment gave similar results (data not shown).

Levels of DNA methylation were also measured, as RPKM, in gene promoters, gene bodies, or intergenic regions (Fig. 4c). Data from un-treated cultures of the 4 Holstein and 4 Angus were combined for this analysis, as there were no significant breed

differences. Levels of methylation in all three regions were significantly different ($P < 0.05$), with gene promoters having the lowest and intergenic regions having the highest levels of methylation.

3.4 Discussion

Variation exists between cows in their innate immune response to mastitis. A greater understanding of the basis for this variation could facilitate new breeding, selection, or management practices to develop animals with superior ability to contain and eliminate pathogens from the normally sterile mammary gland. We have previously used dermal fibroblasts to model this animal variation within groups of Holstein cows (Kandasamy et al., 2011, Benjamin et al., 2015). In these studies we found substantial differences between dairy cows in the magnitude of their fibroblasts' response following treatment with LPS or PAM2CSK4 that are ligands for TLR4 and TLR2/6, respectively. Intra-mammary bacterial challenges were also conducted on groups of lactating animals from these studies that had been classified as having low or high responding phenotypes. Low responding animals developed a reduced inflammatory response characterized by less leakage of bovine serum albumin (BSA) into milk, as well as a less severe reduction in milk quality, as determined by a lower milk somatic cell count. This reduced response was accompanied by similar bacterial clearance rates between the phenotypes, suggesting the heightened response of the high responding animals did not offer any advantage in resolution of the infection. There is limited data to suggest that a high response phenotype is characteristic of dairy in comparison to beef breeds of cattle (Glass et al., 2005b, Piper et al., 2009) and thus, in the current study, we have expanded our use of the

fibroblast model to explore differences between a dairy breed (Holstein) and a beef breed (Angus) in their innate responses to LPS exposure.

Previous studies have looked at differences in the innate immune response between dairy breeds (Holstein and Jersey) following an intra-mammary challenge with *S. aureus* (Bannerman et al., 2008b) or *E. coli* (Bannerman et al., 2008a). While there was high inter-animal variation in milk levels of IL8 and TNF during the challenge, the overall responses were similar between Holstein and Jersey animals. This suggested that the innate immune response is conserved between these two dairy breeds. Likewise, an intravenous LPS challenge comparing beef breeds (Angus and Romosinano) revealed small differences in the serum response profiles of TNF and IL1B (Carroll et al., 2011). Each of these studies examined differences within dairy or within beef breeds, while few studies have explored differences in innate immune responses between a dairy and a beef breed. In one such study, skin biopsies were collected from Holstein (*B. taurus*) and Brahman (*B. indicus*) animals following an experimental tick challenge to compare localized innate immune responses at tick-attachment sites (Piper et al., 2010). Microarray analysis of the biopsies revealed that Holstein animals had greater expression of many innate immune response-associated genes in comparison to the Brahmans. It was suggested that the greater expression of chemokine and cytokines at tick-attachment sites might facilitate feeding by the tick, thereby leading to the lower resistance to tick infestations observed in the Holstein breed. Similarly, Holstein and Sahiwal (*B. indicus)* calves were used to explore breed differences in the inflammatory response (Glass et al., 2005b). In that study, calves from both breeds underwent an experimental challenge with *Theileria annulata,* the protozoan parasite that causes bovine theileriosis. Holstein calves

experienced severe clinical symptoms following the challenge while Sahiwal calves seemed better able to control the inflammatory response. Furthermore, we have previously presented data from a related experiment, now being prepared for full publication, indicating that Holstein heifers have greater serum IL6 and TNF levels after an intravenous LPS challenge compared to age-matched Angus heifers (Benjamin et al., 2014). In our current study, a comparison of Holstein and Angus fibroblasts revealed significant breed differences in response to an LPS challenge, with Holstein cultures exhibiting a hyper-responsive phenotype compared to the hypo-responsiveness of Angus cultures.

3.4.1 Holstein vs. Angus differences in LPS response

RNA-Seq analysis of LPS-challenged fibroblasts revealed a large number of immune-associated differentially expressed genes between Holstein and Angus cultures. Many of these genes are known to play critical roles in the response to — and the recovery from — bacterial infections such as *E. coli* mastitis (Burvenich et al., 2003, Kandasamy and Kerr, 2012). In particular, *TLR4*, which is the extracellular receptor responsible for recognition of LPS, was expressed at higher levels in Holstein cultures before (4.5 fold) and following LPS stimulation (5.7 and 3.1 fold). This may suggest the Holstein animals will detect and respond to Gram-negative bacteria more vigorously than Angus animals. Additionally, several subunits of the NF-κB transcription factor complex were differentially expressed between breeds. Specifically, Holstein cultures had greater expression of *RELB* (1.8-fold) at hours 0 and 2, and *NFKB1* (1.6-fold) and *NFKB2* (1.8-fold) at hour 8 post LPS treatment. This transcription factor is central to innate immune

signaling pathways leading from a diverse array of pattern recognition receptors including the TLR and NOD receptor families (Sun et al., 2013). Once activated, NF-κB promotes the transcription of chemokine and cytokine genes associated with the innate immune system. While the activity of *NFKB* is carefully regulated under normal conditions, excessive production of pro-inflammatory cytokines can lead to its further activation, which may create a chronic inflammatory condition (Hayden and Ghosh, 2012). The higher expression of both *TLR4* and the various subunits of *NFKB* complex in the Holstein fibroblasts would enable the greater responsiveness to LPS observed in this breed.

An appropriate innate immune response is also dependent on timely production of anti-inflammatory factors once the stimulatory agent has been eliminated. The chemokine receptor 4 (CXCR4) and its sole ligand, CXCL12 are important in this regard and their interaction has been shown to suppress *TLR4* signaling and *NFKB* activation in response to LPS in human embryonic kidney cells (Kishore et al., 2005) and to suppress LPS-induced IL6 production by murine bone marrow-derived macrophages (Fan et al., 2012). The CXCL12 chemokine has the ability to activate anti-inflammatory pathways in T-cells and macrophages and to suppress inflammation (Meiron et al., 2008, Karin, 2010). A single injection of a CXCL12 analog administered to mice in parallel with LPS was able to decrease the levels of LPS-induced plasma TNF (Fan et al., 2012). Within our fibroblasts, Angus cultures expressed greater levels of *CXCR4* and *CXCL12* prior to LPS treatment. At hours 2 and 8 post-LPS, *CXCL12* expression continued to be higher in Angus while the differences in *CXCR4* expression disappeared. Due to the inhibitory

function of *CXCR4* and *CXCL12*, the higher basal expression of these two genes in Angus cultures may have reduced their LPS responsiveness.

Breed differences in the expression of genes associated with the maintenance of DNA methylation were also uncovered in RNA-Seq analysis. *DNMT1* and *UHRF1* were more highly expressed in Angus cultures both basally and following LPS exposure. DNMT1 is responsible for maintaining DNA methylation patterns following replication (Moore et al., 2013), and UHRF1 interacts with hemimethylated DNA and recruits DNMT1 to facilitate methylation of the daughter strand (Bostick et al., 2007). Greater expression of these two genes in Angus cultures may indicate this breed is better able to maintain methylation levels. Increased methylation of gene promoter regions is generally associated with reduced gene transcription and thus could account for the reduced expression of pro-inflammatory genes in fibroblasts from this breed.

3.4.2 Methylation differences between breeds

The breed comparison also included an examination into the potential role that differences in early life environment might play in determining breed differences in an animal's epigenome. Holstein calves are removed from their dams within 24 h of birth, moved to individual housing, and fed a milk replacer diet; while Angus calves are allowed to nurse from the dam for months and are typically housed with the herd out on pasture. These environmental differences could affect DNA-methylation patterns and lead to differences in gene transcription in response to immune stimulation. The MIRA-Seq analysis of Holstein and Angus fibroblasts revealed 49 3Kb regions of the genome that were differentially methylated between breeds (Table 3). In a global sense, the breed

difference was minimal despite the higher expression of *DNMT1* and *UHRF1* in Angus cultures. Unexpectedly, genes involved in the LPS-response pathway were under-represented in these 49 regions. While it would appear that DNA methylation does not play a major role in the differential breed response to LPS observed in our fibroblast model, MIRA-Seq is only a moderate-resolution technique and a potential limitation of the current study. The MIRA technique is based on the high affinity of a two-protein complex formed by methyl-CpG-binding proteins (MBD) 2b and 3L1, which bind to methylated DNA in a methylated CpG density-dependent manner (Jung et al., 2015). Regions of fragmented DNA with greater methylated CpG content, such as CpG islands, will have enhanced affinity for the MBD complex, allowing for enrichment of these regions in the final analysis, whereas regions with low CpG content will be under-represented. While this allows for a comparison of the overall methylome between individuals, MIRA-Seq is not sensitive enough to detect more subtle, but influential, differences in methylation at specific CpG sites. For example, differential methylation at a single CpG site in the *IL6* gene promoter was attributed to differential serum levels of IL6 in patients suffering from rheumatoid arthritis (Ishida et al., 2012). Patients that were experiencing elevated serum levels of IL6 had lower methylation rates at a single CpG site in the gene promoter, suggesting that methylation at this site represses *IL6* transcription. Subtle differences in CpG methylation between Holstein and Angus cultures may have been lost in our analysis. Future studies of methylation differences between cattle breeds would benefit from a technology with greater resolving power such as those based on sequencing of bisulfite converted DNA (Mensaert et al., 2014).

3.4.3 Identification of biomarkers linked to phenotypic responses

Previously, we have demonstrated age-dependent increases in LPS-responsiveness of fibroblasts collected from the same animals at 5 and 16 months of age (Green et al., 2011). Cultures isolated from young (5-month-old) Holsteins produced considerably lower levels of IL8 following LPS exposure compared to cultures collected from the older (16-month-old) Holsteins. RNA-Seq analysis was completed on the young vs. old cultures following LPS treatment, and revealed several pro-inflammatory response genes, including *IL8*, *IL6*, *CCL20*, *TNF*, and *CXCL2* that had greater expression in older cultures following LPS stimulation (Green et al., 2015). Comparing the RNA-Seq data sets from the previous young vs. older Holstein study, and the current Holstein vs. Angus study revealed several genes that had similar expression patterns defining a high vs. low response phenotype. The higher response phenotype of older animals in the previous study, and Holstein animals in the current study, is characterized by greater expression of *TLR4, IL8, CCL2, CCL5, CCL20*, and *TNF*. These genes are critical in the recognition of, and resulting inflammation following infection by Gram-negative bacteria. The identification of this set of biomarkers from the global transcriptome may allow for greater precision in classifying an animal's innate immune response phenotype even at a young age. This has potential utility in selecting low response replacement heifers as young calves to avoid raising high response animals that are more likely to develop severe rather than milder forms of coliform mastitis. Alternatively, selective modification of the Holstein genome with appropriate Angus genes may be used to generate Holstein animals with a reduced, potentially beneficial, innate response to infection.

3.5 Conclusions

Our previous work on characterizing within-breed variation in the innate immune response has now been extended to reveal substantial differences between a dairy and a beef breed. Our fibroblast model system indicates that divergent selection for dairy or beef character has resulted in substantial differences in immune phenotypes between these breeds, with dairy animals being more responsive to LPS than beef animals. Genetic differences between the dairy and beef breeds are likely of key importance, but management differences resulting in differing *in utero* and neonatal environments may lead to epigenetic differences as well. Events such as health of the dam during pregnancy or the level of maternal care a calf receives in early life may lead to epigenetic modifications that alter that animal's innate immune response phenotype as an adult. Our current studies also provide a set of gene expression biomarkers, measurable in dermal fibroblasts, which are indicative of an animal's innate immune response magnitude.

3.6 Materials and Methods

3.6.1 Animals and experimental set-up

Female animals of two cattle breeds were used in this study: Angus (n = 12) and Holstein (n = 5). All animals were 19.4 (± 0.1) months of age at the time of skin sampling (see below) and were housed and cared for at the University of Minnesota. The Institutional Animal Care and Use Committee at both the University of Vermont and the University of Minnesota approved all animal procedures prior to beginning the study.

3.6.2 Dermal Fibroblast Isolation

Skin biopsies were collected from the shoulder area of animals housed at the University of Minnesota as previously described (Kandasamy et al., 2011) and shipped overnight on ice packs to the University of Vermont in a transport media consisting of 1X Dulbecco's PBS (DPBS; Hyclone Laboratories, Logan, UT) with 1X antibiotic cocktail (100 U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin B; Hyclone Laboratories). Once received, biopsy samples were processed as described (Benjamin et al., 2015). Briefly, fibroblasts were isolated with a 0.5% collagenase type I enzyme solution (Life Technologies, Grand Island, NY) and seeded in a 25cm^2 flask (Corning Inc., Corning, NY) in Dulbecco's Modified Eagle Medium (DMEM; Hyclone Laboratories) with 10% FBS (Hyclone Laboratories), 1X antibiotic cocktail, and 1X Insulin-Transferrin-Selenium (ITS; Mediatech Inc., Herndon, VA). Upon confluency, cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded into a 75cm^2 flask (Corning Inc.) in DMEM with 5% FBS, 1X antibiotic cocktail, and 1X ITS. After approximately four days, cells were expanded into three 75cm^2 flasks, and once confluent, cells were detached from the flasks with trypsin, diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma- Aldrich, St. Louis, MO), and nine aliquots of third passage cells were cryopreserved in liquid nitrogen for subsequent challenges.

3.6.3 In vitro challenges

A challenge was conducted to compare breed differences in the fibroblast response to LPS exposure on fibroblasts isolated from Holstein and Angus animals.

Aliquots of fibroblasts isolated from both breeds were revived in parallel from cryopreservation and cultured in a 75cm^2 flask in DMEM supplemented with 5% FBS, 1X antibiotic cocktail, and 1X ITS. Once confluency was reached, cells were detached with 0.25% trypsin, washed, counted with a cell counter (Bio Rad, Hercules, CA) and seeded into 6-well plates (Corning Inc.) at 1.25 x 10^5 cells/ml in a total volume of 2ml. Media was replaced 24 h later with 2ml of either fresh media (negative control), media containing 100ng/ml of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich), or media containing 1ng/ml of recombinant bovine IL1B (AbD Serotech, Raleigh, NC). After the challenge period, media was collected from each well, spun at 10,000x *g* for one minute to remove cell debris, and stored at -20°C until further analysis.

3.6.4 IL8 ELISA

The concentration of IL8 in conditioned media samples was determined by a custom sandwich ELISA as described previously (Kandasamy and Kerr, 2012). Mouse anti-bovine (clone 170.13, gifted by Samuel Maheswaren, University of Minnesota, St. Paul, MN) and a biotinylated goat anti-human IL8 (R&D Systems Inc., Minneapolis, MN) were used as capture and detection antibodies, respectively. Recombinant bovine IL8 (Thermo Scientific, Rockford, IL) was used as the assay standard. The detection limit for this assay was 130pg/ml. Differences in IL8 protein production between Holstein and Angus fibroblast cultures were determined using a Student's *t*-test (Graph Pad Prism 6.0).

3.6.5 RNA-Seq

Fibroblast cultures collected from four animals of each breed (Holstein and Angus) were randomly chosen for investigation of whole transcriptome (RNA-Seq)

differences between the breeds. Aliquots from each culture were revived from cryopreservation and grown to confluency in a 75cm^2 flask with DMEM supplemented with 5% FBS, 1X antibiotic cocktail, and 1X ITS. Cells were lifted with 0.25% trypsin, counted, and seeded into 6-well plates at 1.00 x 10^5 cells/ml. Following a 48 h incubation, fibroblasts were challenged with 100ng/ml of LPS and RNA was collected from replicate wells at three time points: hour zero (control conditions), hour two, and hour eight post-LPS using the PurefectPure RNA Cultured Cell extraction kit (5 Prime, Hamburg, Germany), which includes a DNase treatment step to eliminate DNA contamination. RNA concentration and quality were assessed using a Qubit Spectrofluorometer (Life Technologies, Carlsbad, CA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), ensuring that all samples had an RNA integrity number (RIN) of 9.5 or greater. Libraries for RNA-Seq analysis were constructed as previously described (Green et al., 2015). Briefly, 500ng of total RNA was PolyA enriched using magnetic beads, reversed transcribed, and the resulting cDNA was fragmented, end repaired, and adenylated. Oligonucleotide adaptors (Illumina, San Diego, CA), each with a unique adaptor sequence or barcode, were then ligated onto each sample. PCR amplification was completed using Illumina reagents, followed by quality assessment (as described above), and high accuracy qPCR quantification (KAPA Biosciences kit # 4824, Barre, VT). AMPure XP Magnetic Beads (Beckman Coulter, Pasadena, CA) were used in cDNA clean-up steps. Sequencing was performed using a 12pM/flow cell lane on an Illumina CBOT for flow cell cluster generation and the Illumina HiSeq1000 for sequencing by synthesis equipped with the HiSeq Control and sequence analysis software.

3.6.6 DNA isolation and methylated CpG Island recovery assay (MIRA-Seq)

MIRA-Seq libraries were generated to investigate genome wide methylation levels in fibroblasts isolated from Holstein and Angus cattle. Genomic DNA was isolated from the same Holstein (n = 4) and Angus (n = 4) cultures used in the RNA-Seq experiment (with no exposure to LPS) using the 5 Prime Pure Perfect Archive DNA Extraction kit (Hamburg, Germany). Following extraction, genomic DNA was sonicated and processed into MIRA-Seq libraries of approximately 300-700 bp in size, essentially as described previously [35] starting with 1.5µg of DNA. MIRA pull-down was performed using the Methyl Collector Ultra Kit (Active Motif, Carlsbad CA) per manufacturer's instructions. Sequencing of the libraries was performed using a 12pM/lane bridge amplification on an Illumina CBOT for flow cell cluster generation and the HiSeq1000 for sequencing by synthesis equipped with the HiSeq Control and sequence analysis software.

3.6.7 Analysis of RNA-Seq data

Raw sequence reads that had a median quality (Q) score of less than 20, more than 3 uncalled bases, or were less than 25bp following trimming were removed from further analysis, and filtered reads were aligned to the reference UMD v3.1 (Zimin et al., 2009) bovine genome using the software package NextGENe v. 2.3.4 (Softgenetics, State College, PA). In order for a read to be considered a mapped read, alignment parameters required > 85% of the read's length to align to the reference sequence. After reads were mapped using NextGENe, total raw read counts were generated for each annotated gene, as defined by the UCSC genome browser UMD v 3.1/bosTau 6.

RNA-Seq data was analyzed by statistical methods used by edgeR in the R software package (version 3.0.1). Initially, genes with a low read count, defined as at least one mapped read per million mapped reads (counts per million; CPM) in less than 50% of the samples being compared, were eliminated. For example, comparison of cultures from Holstein and Angus animals at a given time point required analysis of n=4 samples/breed, so at least 4 samples needed a CPM equal to or greater than 1 to be considered for analysis.

A generalized linear model likelihood-ratio test using the limma package was used to conduct comparisons of cultures from animals of the two breeds at the different time points post-LPS for RNA-Seq. Similar analysis was also employed to determine the LPS response (0 h vs. 2 h and 0 h vs. 8 h post-LPS). Raw p-values were adjusted to account for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

When analyzing the effect of breed on the LPS response, genes were considered differentially expressed if they passed the false discovery rate (FDR) < 0.05 and fold change (FC) ≥ 2 thresholds. The effect of LPS on gene expression was determined by comparing cultures at the 2- and 8-h time points to the 0 h cultures. The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009b, a) was used for functional annotation and analysis by uploading the official gene symbol of statistically significant genes (FC ≥ 2; CPM > 1; FDR < 0.05 for RNA-Seq).

3.6.8 Analysis of MIRA-Seq

Raw sequence reads were filtered and aligned as described above. After reads were mapped using NextGENe, total raw read counts for each gene, as defined by the UCSC genome browser, UMD v 3.1/bosTau6, were generated. Data analysis was completed using the edgeR module in the R software package (v3.0.1). Genes with a low read count, defined as a CPM of at least 1 in less than 50% of the samples were removed. A generalized linear model likelihood-ratio test using the limma package was employed to compare methylation levels between cultures from Holstein and Angus animals. Identification of differentially methylated regions (DMR) was completed on 3Kb windows of the genome to allow for an overall assessment of the bovine fibroblast methylome. Raw p-values were adjusted to account for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). For analysis of MIRA-Seq data, levels of methylation were considered different between breeds if the region passed the FDR < 0.1 and FC ≥ 2 thresholds.

3.6.9 Quantitative real-time PCR

Five immune-associated genes from those identified in RNA-Seq were selected for quantitative real-time PCR (RT-qPCR). Oligonucleotide primers specific for the following genes were used: *Toll-like receptor 4* (*TLR4*), *interleukin 8 (IL8)*, *tumor necrosis factor alpha* (*TNF*), *chemokine (C-C-motif) ligand 5* (*CCL5*), and *chemokine (C-C-motif) ligand 20* (*CCL20*). Primer sequences are in Table 4. The RNA that was used for the whole transcriptome analysis was also used in RT-qPCR. The Improm II Reverse Transcriptase kit (Promega, Madison, WI) was used to complete first strand cDNA

synthesis. Gene expression was quantified by RT-qPCR on a CFX96 Real-Time Instrument (Bio-Rad) using PereCTa SYBR Green Super-Mix, Low ROX kit (Quanta Biosciences). Cycling conditions were: initial denaturation at 95°C for 2 minutes; then 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Samples were run in duplicate and melt curves were performed to check amplification of desired gene product. The *beta-actin* (*ACTB*) gene was used as a reference gene for normalization (Robinson et al., 2007, Bougarn et al., 2011b). Cycles to threshold (Ct) were calculated for each sample and analyzed with the ΔCt method with fold change being $2^{-\Delta\Delta Ct}$. Analysis of RT-qPCR data was completed using a two-way ANOVA model with repeated measures (Graph Pad Prism 6.0). Comparisons with $P < 0.05$ were considered statistically significant within experiments.

3.6.10 Relationship between MIRA-Seq and RNA-Seq

To determine a relationship between DNA methylation and gene expression, the average reads per kilobase per million matched reads (RPKM) from the RNA-Seq and MIRA-Seq of the 8 cultures from the two cattle breeds were investigated. Gene body and intergenic regions were determined based on annotations of the UMD v3.1 bovine genome, while gene promoters were defined as -2500 to +500 bp from the gene transcription start site. A two-tailed Fisher's exact test in the R software package was used to determine the relationship between mRNA transcription levels and DNA methylation, in which, an association was investigated between low or high levels of methylation and either low or high gene expression. Values for gene expression RPKM were calculated as the cumulative size of the gene exons, while for gene methylation,

gene body length was the total size of both intronic and exonic segments. All values were normalized to library and transcript size by conversion of read counts into RPKM values. RNA-Seq RPKM values were binned into either low or high levels at a cutoff of RPKM = 5, while MIRA-Seq RPKM values were divided into low and high levels at PRKM = 0.5.

To determine if the type of genomic region had an effect on DNA methylation levels, average RPKM was calculated for gene promoters, gene bodies, and intergenic regions. Gene body and intergenic regions were determined by UMD v3.1 bovine genome, and gene promoters were defined as -2500 to +500 bp of a gene transcription start site. A one-way ANOVA with a Bonferroni post-test for multiple comparisons was run to determine differential methylation levels based on genomic location.

3.7 Availability of supporting data

The datasets supporting the conclusions of this article are available in NCBI's Gene Expression Omnibus and are accessible through GEO SuperSeries accession number GSE72075 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72075.

3.8 Additional files

Additional file 1: Genes displaying differential gene expression (FDR < 0.05; CPM > 1; 2 ≤ FC ≤ -2) due to LPS at hour 2 as compared to hour 0. A positive fold change indicates higher expression at hour 2 compared to hour 0. CPM = counts per million. FDR = false discovery rate. Data shown for comparisons with an FDR < 0.05; CPM > 1; FC ≥ 2.

Additional file 2: Genes displaying differential gene expression (FDR < 0.05; CPM > 1; 2 ≤ FC ≤ -2) due to LPS at hour 8 as compared to hour 0. A positive fold change indicates higher expression at hour 8 compared to hour 0. CPM = counts per million. FDR = false discovery rate. Data shown for comparisons with an FDR < 0.05; CPM > 1; FC ≥ 2.

Additional file 3: Differentially expressed genes (FDR < 0.05; CPM > 1; 2 ≤ FC ≤ -2) between Holstein and Angus fibroblast cultures exposed to 100ng/ml LPS for 0, 2, and 8 hours. A positive fold change indicates higher expression in Holstein cultures. CPM = counts per million. FDR = false discovery rate

***Please obtain additional files from Dr. David Kerr (David.Kerr@uvm.edu) ***

Competing interests

The authors declare that they have no competing interests.

Author contributions

AB assisted in experimental design, performed in vitro experiments, acquired and analyzed data from MIRA- and RNA-Seq, completed RT-qPCR, and drafted the manuscript. BG assisted in the MIRA- and RNA-Seq data analysis. SM assisted in the development of the MIRA-Seq protocol and analysis and provided editorial comments. BC provided the skin biopsy samples and contributed to edits of the manuscript. DK assisted in experimental design and assisted in data analysis and manuscript edits. All authors read and approved the final manuscript.

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Table 1 Average LPS response from combined Holstein and Angus cultures measured by increases in expression of immune-associated genes compared to hour 0 post-LPS.

Gene	Gene Name	Fold Change*	
Transcription and activation pathways		Hour 2	Hour 8
BIRC3	Baculoviral IAP repeat-containing protein 3	7.3	4.2
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in	24.3	9.3
NFKBIZ	Nuclear factor of kappa-B inhibitor zeta	11.7	3.2
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in	- -	2.8
NFKBID	Nuclear factor of kappa light polypeptide gene enhancer in	2.3	- -
Cytokines, chemokines, and growth factors			
CCL2	Chemokine (C-C motif) ligand 2	14.9	14.6
CCL5	RANTES	49.6	132.8
CCL20	Chemokine (C-C motif) ligand 20	1102.1	3147.3
CXCL2	Chemokine (C-X-C motif) ligand 2	67.9	31.6
CXCL6	Chemokine (C-X-C motif) ligand 6	12.3	45.0
IL1A	Interleukin 1, alpha	9.2	5.7
IL6	Interleukin 6	88.2	124.1
IL8	Interleukin 8	325.7	387.8
SAA3	Serum Amyloid A 3	65.1	1145.6
TNF	Tumor Necrosis Factor, alpha	87.4	- -
Type I IFN-related genes			
IRF1	Interferon regulatory factor 1	12.3	4.2
IRF5	Interferon regulatory factor 5	3.0	- -
ISG15	Ubiquitin-like modifier	- -	308.3
MX2	Myxovirus (influenza virus) resistance 2	- -	156.6
OAS1	2'-5'- oligoadenylate synthetase 1	- -	128.7
OAS2	2'-5'- oligoadenylate synthetase 2	- -	65.6

*Data obtained by RNA-Seq and presented as fold induction of the indicated gene at either 2 or 8 hours post-LPS in comparison to expression levels at 0 hours post-LPS. All fold changes shown are FDR<0.05; FC>2; and CPM >1. - - indicates FDR>0.05, FC<2, or CPM<1.

Table 2 Differential gene expression (fold change) of immune-associated genes at hours 0, 2, and 8 post-LPS treatment of fibroblasts collected from Holstein animals as compared to fibroblasts collected from Angus animals.

Gene	Hour 0	Hour 2	Hour 8
Innate Immune Related Genes			
CCL2	- -	- -	2.9
CCL5	- -	- -	5.7
CCL20	- -	- -	8.6
CCL26	- -	2.8	3.6
CX3CL1	- -	- -	14.8
CXCL12	-5.7	-7.4	-6.5
CXCL16	- -	- -	2.3
CXCR4	-3.1	- -	- -
HMGB1	-1.9	-1.9	-1.8
IFIH1	- -	- -	3.6
IL1R1	- -	1.5	- -
IL8	- -	- -	4.5
IRF5	2.4	2.4	2.6
MX2	- -	- -	14.0
NOD1	- -	2.4	- -
TLR2	-3.5	-2.8	- -
TLR4	3.0	4.0	2.5
TLR6	1.8	- -	2.5
TNF	- -	6.9	- -
TNFSF18	6.5	6.0	5.1
TNFSF4	4.4	- -	2.6
TRAF1	2.4	2.4	- -
TRAIP	- -	-2.7	- -
DNA Methylation Associated			
DNMT1	-1.6	-1.5	-1.5
DNMT3A	- -	- -	- -
DNMT3B	- -	- -	- -
UHRF1	-2.3	-2.0	-2.0

Data obtained by RNA-Seq and presented as fold change between Holstein and Angus cultures. Positive values indicate higher expression in Holstein cultures as compared to Angus cultures. All presented values indicate FDR<0.05 with an average CPM> 1, while - - notes a values with either FDR>0.05 or an average CPM< 1.

Table 3 Differentially methylated 3Kb regions between Holstein and Angus cultures as determined by MIRA-Seq and gene expression data for annotated gene.

				RNA-Seq fold change***		
FC*	Chr	Genomic coordinates	Gene**	Hour 0	Hour 2	Hour 8
-2.9	1	8811701 - 8814700	**ADAMTS5**	- -	- -	- -
4.2	1	4686501 - 4689500	LOC100848874	- -	- -	- -
2.0	1	8325701 - 8328700	LOC526789	- -	- -	- -
-3.4	2	88463334 - 88466333	- -	- -	- -	- -
-3.4	2	88469334 - 88472333	- -	- -	- -	- -
2.6	2	18491734 - 18494733	OSBPL6	- -	1.4	1.5
2.0	5	116626706 - 116629705	FBLN1	- -	- -	- -
2.3	6	5529182 - 5532181	- - -	- -	- -	- -
-8.3	7	18532846 - 18535845	LOC100337044	- -	- -	- -
2.9	8	23178787 - 23181786	LOC100300143	- -	- -	- -
-2.6	8	34929387 - 34932386	- -	- -	- -	- -
-2.7	8	78669587 - 78672586	- -	- -	- -	- -
-2.8	8	12568687 - 12571686	- -	- -	- -	- -
-4.0	8	79335787 - 79338786	**NTRK2**	- -	- -	- -
-2.6	8	98801087 - 98804086	- -	- -	- -	- -
-2.6	9	20444051 - 20447050	- -	- -	- -	- -
-2.7	9	93571151 - 93574150	- -	- -	- -	- -
-4.0	9	88292251 - 88295250	ULBP1	- -	- -	- -
-2.7	10	32127701 - 32130700	- -	- -	- -	- -
2.1	12	51081922 - 51084921	- -	- -	- -	- -
-2.5	13	64236797 - 64239796	ASIP	-14.0	-15.2	-13.4
2.8	13	48576797 - 48579796	LRRN4	- -	- -	- -
2.6	14	36500947 - 36503946	- - -	- -	- -	- -
2.1	15	53581057 - 53584156	LOC100336675	- -	- -	- -
-2.3	15	9012857 - 9015856	- -	- -	- -	- -
-7.5	15	27535857 - 27538856	- -	- -	- -	- -
-2.8	15	55516557 - 55519556	SERPINH1	- -	- -	- -
-2.4	17	7224294 - 7227293	MAB21L2	- -	- -	- -
2.2	17	17977294 - 17980293	MAML3	- -	- -	- -
-2.8	18	60220698 - 60223697	LOC100848332	- -	- -	- -
-3.6	18	63217498 - 63220497	LOC783134	- -	- -	- -
2.4	18	33452798 - 33455797	- -	- -	- -	- -

(Table 3 continued)

				RNA-Seq fold change***		
FC*	**Chr**	**Genomic coordinates**	**Gene****	**Hour 0**	**Hour 2**	**Hour 8**
2.4	19	42556475 - 42559474	- -	- -	- -	- -
2.3	19	62858675 - 62861674	AXIN2	- -	- -	- -
-2.4	19	43381475 - 43384474	CNTNAP1	- -	1.8	2.0
-3.6	19	23655275 - 23658274	HIC1	- -	1.7	- -
2.2	19	42109475 - 42112474	KRTAP9-2	- -	- -	- -
2.4	19	62705675 - 62708674	RGS9	2.0	2.1	2.5
4.8	23	49777993 - 49780992	- - -	- - -	- - -	- - -
2.8	24	59112931 - 59115930	CCBE1	-2.3	-2.2	-1.8
2.4	24	62388931 - 62391930	- - -	- - -	- - -	- - -
2.5	25	27266601 - 27269600	- - -	- - -	- - -	- - -
2.4	26	23658031 - 23661030	C26H10orf26	- - -	- - -	1.4
-2.4	26	16776031 - 16779030	LOC100848660	- - -	- - -	- - -
3.2	27	23720367 - 23723366	- - -	- - -	- - -	- - -
-2.4	X	63726195 - 63729194	LOC100848206	2.4	2.6	2.3
2.1	X	111529495 - 111532494	LOC516666	- - -	- - -	- - -
2.1	X	128195395 - 128198394	- - -	- - -	- - -	- - -
-2.6	X	55935095 - 55938094	- - -	- - -	- - -	- - -

*Data obtained by MIRA-Seq and presented as fold difference in read count of methylated regions. Positive fold change indicates higher methylation levels in Holstein cultures while negative values are higher methylation in Angus cultures. ** DMRs with an associated gene indicate that some portion of the 3Kb region falls within an annotated gene, while - - indicates DMRs that are intergenic. Bolded gene names indicate that the discovered DMR fell within the promoter region of that gene (-2500 to +500bp from gene transcription start site). *** Positive values indicate greater expression in Holstein cultures as compared to Angus.

Table 4 RT-qPCR primer pairs used for amplification of target genes.

(The top and bottom sequences are the forward and reverse, respectively)

Gene	Primer Sequences	Reference
TLR4	ACTGCAGCTTCAACCGTATC TAAAGGCTCTGCACACATCA	Ibeagha-Awemu et al., 2008
IL8	GCTGGCTGTTGCTCTCTTG AGGTGTGGAATGTGTTTTTATGC	Pareek et al., 2005
TNF	TCTTCTCAAGCCTCAAGTAA CCATGAGGGCATTGGCATAC	Bougarn et al., 2015
CCL20	TTCGACTGCTGTCTCCGATA GCACAACTTGTTTCACCCACT	Gilbert et al., 2013
CCL5	CTGCCTTCGCTGTCCTCCTGATG TTCTCTGGGTTGGCGCACACCTG	Gilbert et al., 2013
ACTB	GCAAATGCTTCTAGGCGGACT CAATCTCATCTCGTTTTCTGCG	Pareek et al., 2005

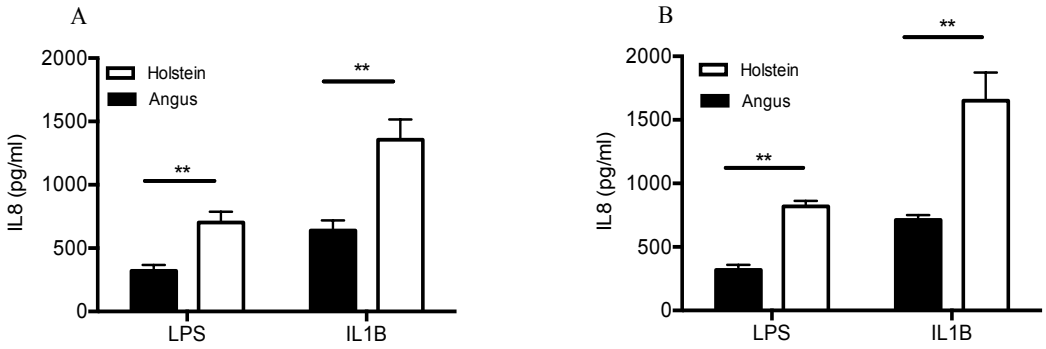

Figure 1 IL-8 protein production from Holstein and Angus fibroblasts.

IL8 production by dermal fibroblasts isolated from 19-month old Holstein (n=5) and Angus (n=12) heifers and challenged for 24 h with LPS (100ng/ml) or IL1B (1ng/ml) (A). Eight cultures (n=4/breed) were randomly chosen for RNA-Seq and MIRA-Seq analysis and levels of IL8 protein following second 24 h challenge with either LPS (100ng/ml) or IL1B (1ng/ml) on these 8 cultures are shown in (B). There was no detectable IL8 production from un-treated cultures of both breeds. Values are mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$.

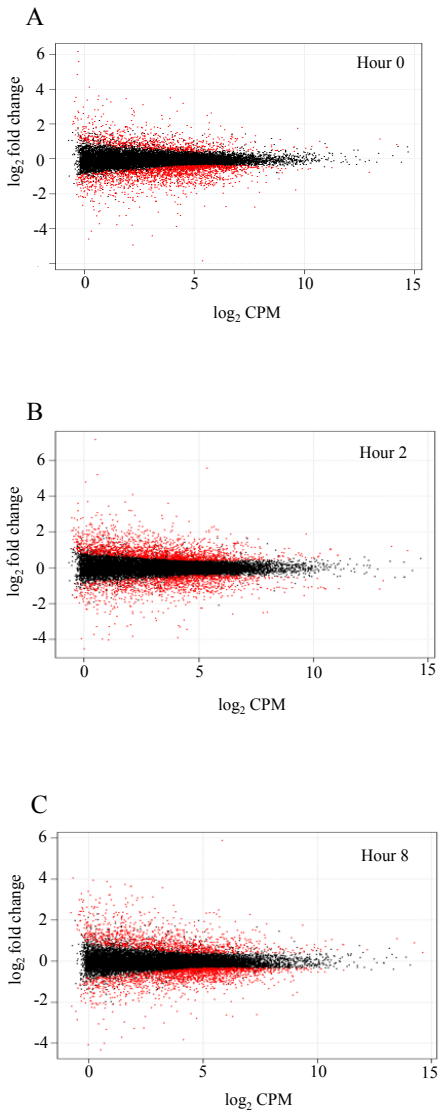

Figure 2 Scatter plots of RNA-Seq analysis.

Eight fibroblast cultures (four of each breed) were used in RNA-Seq to explore gene expression differences. Scatter plots of indices analyzed from RNA-Seq data were generated for expression level (log_2CPM) and differential expression (log_2 Fold Change) at 0 hours (A), 2 hours (B), and 8 hours (C) following LPS exposure. Positive fold change values indicate higher expression in Holstein cultures while negative fold change values show higher expression in Angus cultures. Red dots denote FDR < 0.05.

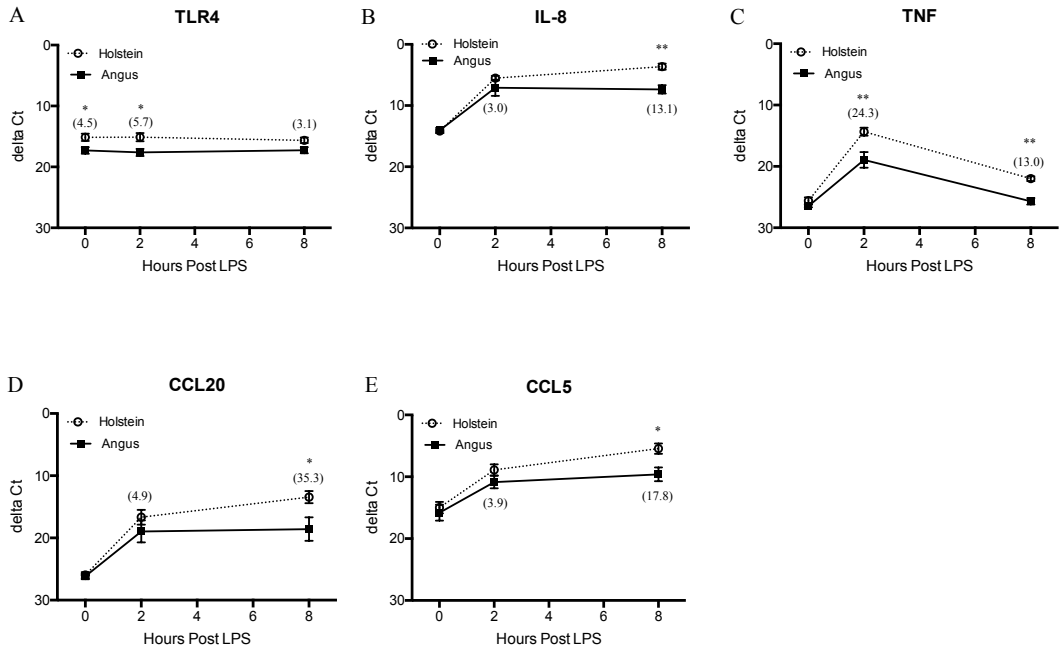

Figure 3 Expression of selected genes by Holstein and Angus fibroblasts.

Differences in expression of *TLR4* (A), *IL8* (B), *TNF* (C), *CCL20* (D), and *CCL5* (E) between Holstein and Angus fibroblast cultures at hours 0, 2, and 8 post-LPS exposure were determined by RT-qPCR. Values are expressed as delta Ct, or the difference in cycles to threshold (Ct) between the gene of interest and the endogenous gene control beta-actin (*ACTB)*. Fold differences ($2^{-\Delta\Delta Ct}$) in expression between Holstein and Angus cultures are indicated in parentheses. All values are mean ± SEM. (n=4/group). *P < 0.05 and **P < 0.01 (t-test).

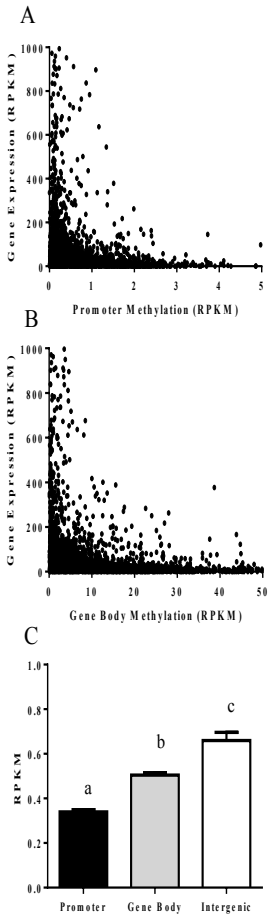

Figure 4 Role of DNA methylation on gene expression and differential methylation levels due to genomic location.

As there were no significant effects of breed on the correlations between genomic location of DNA methylation and subsequent gene expression, data was combined for the 4 Holstein and 4 Angus cultures in this analysis. Scatter plots show the relation between gene expression at 8 hours post-LPS treatment and levels of DNA methylation at the gene's promoter (A) or gene body (B). Gene expression levels (RNA-Seq) and DNA methylation (MIRA-Seq) were normalized to gene size and presented as RPKM. The average methylation levels of combined Holstein and Angus MIRA-Seq libraries based upon genomic region (C). Lettering denotes differential methylation levels as measured by a one-way ANOVA with Bonferonni post-test ($p < 0.05$). All values are mean ± SEM.

Chapter 4: Neonatal LPS exposure does not diminish the innate immune response to a subsequent LPS challenge in Holstein bull calves.

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4.1 Abstract

The innate immune response following experimental mastitis is quite variable between individual dairy cattle. An inflammatory response that minimizes collateral damage to the mammary gland while still effectively resolving the infection following pathogen exposure is beneficial to dairy producers. The ability of an LPS exposure in early life to generate a low-responding phenotype and thus reduce the inflammatory response to a later life LPS challenge was investigated in neonatal bull calves. Ten Holstein bull calves were randomly assigned to either an early life LPS (ELL) group (n=5) or an early life saline (ELS) group (n=5). At 7 days of age, calves received either LPS or saline, and at 32 days of age, all calves were challenged with an intravenous dose of LPS to determine the effect of the early life treatment (LPS or saline) on the immune response generated towards a subsequent LPS challenge. Dermal fibroblast (DF) and monocyte-derived macrophage (MDM) cultures from each calf were established at age 20 and 27 d, respectively, to model sustained impacts from the early life LPS exposure on gene expression and protein production of components within the LPS response pathway. The ELL calves had greater levels of plasma IL-6 and TNF-α than the ELS calves following the early life LPS or saline treatments. However, levels of these two immune markers were similar between ELL and ELS calves when both groups were subsequently challenged with LPS. A comparison of the in vitro LPS-responses of the ELL and ELS calves revealed similar patterns of protein production and gene expression following an LPS challenge of both DF and MDM cultures established from the treatment groups. While an early life exposure to LPS did not result in a dampened inflammatory response towards a later LPS challenge in these neonatal bull calves, the potential that exposure to

inflammation or stress in early life or in utero can create an offspring with a low-responding phenotype as an adult is intriguing and has been documented in rodents. Further work is needed to determine if an inflammatory exposure in utero in a dairy animal would result in a low-responding innate immune phenotype.

Keywords: early life LPS, inter-animal variation, inflammatory response

4.2 Introduction

The innate immune system plays a key role in the clearance of pathogens following an infection such as bovine mastitis. This acute, non-specific response is initiated when conserved pathogen associated molecular patterns (PAMP) from a diverse number of pathogens are recognized by germ-line encoded receptors known as pattern recognition receptors (PRR) that are expressed on a variety of cell types. Lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria, a major causative agent of bovine mastitis, is recognized by the PRR toll-like receptor (TLR) 4. Once LPS is bound by TLR4, an intracellular signaling cascade activates transcription factors such as nuclear factor-kappa B (NF-κB) which promote the transcription of cytokines including interleukin (IL) -1β, -6, and tumor necrosis factor alpha (TNF-α) (Kawai and Akira, 2007). These three cytokines play major roles in driving the pro-inflammatory response by promoting febrility, inflammation, and activation of leukocytes to aid in the clearance of the pathogen (Bannerman 2009). However, an excessive inflammatory response can be detrimental to the host, causing collateral damage to tissues, such as the mammary gland (Schukken et al., 2011), thereby reducing future milk production and profit (Grohn et al., 2004). The factors that lead to a more robust inflammatory response

in some animals and not in others have not been fully elucidated. Even in controlled intra-mammary challenge models of mastitis, inter-animal variation has been observed in cytokine concentrations in milk or blood, milk bacterial counts, and severity of clinical symptoms (Schukken et al., 1999, Lee et al., 2006, Simojoki et al., 2009), supporting the belief that host factors play a major role in determining the severity and outcome of mammary infections (Burvenich et al., 2003).

Genetic polymorphisms in components of the host's pathogen recognition pathways could lead to the variable immune responses between animals. Within cattle, a number of single nucleotide polymorphisms (SNP) have been identified in genes encoding the toll-like receptors (Novak, 2014) and SNPs in these and other genes involved in the inflammatory response have been associated with individual differences in their resistance to mastitis (Thompson-Crispi et al., 2014). Epigenetic modifications of an animal's DNA can also contribute to the observed phenotypic differences in the immune response of individuals. DNA methylation in particular, has been shown to influence transcription of genes (Moore et al., 2013). In human diseases such as periodontitis and rheumatoid arthritis, aberrant methylation within immune response genes has been linked to an increased risk of disease or severity of symptoms (Benakanakere et al., 2015, Kojima et al., 2015). The role that epigenetics may play in modulating the bovine innate immune response is now being investigated (Paibomesai et al., 2013, Green and Kerr, 2014, Chang et al., 2015) and may provide a more complete picture of how genetic and epigenetic factors combine to produce inflammatory responses following bovine mastitis that range from mild to severe.

While pathogen detection by innate immune cells prompts a robust inflammatory response that is essential to resolving the infection, this response must be under tight regulation to prevent excessive inflammation and extensive tissue damage. There are some mechanisms that can generate a low responding phenotype in an animal, either transiently or long-term, following pathogen exposure. An animal exposed to a low level of LPS can develop a short-term reduction or even an abolished inflammatory response towards a subsequent LPS exposure, thereby exhibiting endotoxin tolerance (Biswas and Lopez-Collazo, 2009). It has been suggested that this toleraized state, generated primarily through macrophages and their reduced production of TNF-α, could be due to a variety of events, including methylation of histone tails, which modify the chromatin structure of anti- and pro-inflammatory genes (El Gazzar et al., 2007), up-regulation of anti-inflammatory or inhibitory genes (Fan and Cook, 2004), and micro-RNA regulation of immune response genes (Baltimore et al., 2008). Additionally, there is abundant evidence that exposure to inflammation or disease in early life can have long-term consequences on the innate immune response of an animal. Several studies completed on rodents have shown that a neonatal LPS exposure can cause a reduction in the cytokine or febrile response following a LPS challenge in the adult rat (Ellis et al., 2005, Spencer et al., 2011). Thus, it would appear that an early life exposure to LPS has the potential to modify the inflammatory response in the adult animal, creating a low-responding phenotype.

In this current study, we investigated if a single exposure to LPS during the neonatal period would modify the in vivo inflammatory response to a subsequent LPS challenge in dairy calves. Additionally, two cellular models, dermal fibroblasts and

monocyte-derived macrophages, were utilized to explore if early life exposure to LPS would result in sustained epigenetic modifications of the LPS response of these cells, thereby influencing the innate immune response.

4.3 Materials And Methods

4.3.1 Experimental design and animal use

Holstein bull calves that were free of clinical disease and had received 4 L of pooled colostrum within 1 h of birth and a second feeding of pooled colostrum 12 h later were purchased from a collaborating dairy farm. The calves (n=10) were brought to the University of Vermont Miller Research Farm at 3.3 ± 0.5 days of age and given a four-day acclimation period. Calves were housed in individual plastic calf hutches and had access to a fenced-in outside area of approximately 3 m x 3 m. Free choice water and a commercially available calf starter were offered to each calf throughout the trial. Calves were bucket-fed an ad libitum amount of a 23% protein, 22% fat milk replacer (OptiMilk Nature's Formula, Arctic Blend; Poulin Grain, Newport, VT) divided between two feedings per day throughout the study period. The University of Vermont Institutional Animal Use and Care Committee approved of all experimental procedures prior to the commencement of this study.

4.3.2 Early life treatment

Calves were randomly divided into two groups: Early Life LPS (ELL) and Early Life Saline (ELS), each consisting of five calves. At approximately 7 days of age, roughly 2 h after the morning feeding, the early life treatment was administered to each

calf. Calves in the ELL group received a jugular injection of 0.5 ug of LPS/kg of body weight isolated from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO) diluted in saline, while the calves in the ELS group received an equal volume of saline. The dose of LPS was based on our previous experiments with 14 month-old heifers (Green et al., 2011). Prior to a single injection of either the LPS solution or saline, the fur along the jugular vein area was clipped and the area was thoroughly cleansed with 70% ethanol. Rectal temperatures were collected immediately prior to the injection (hour 0) and hourly for seven hours. Blood samples were collected at hours 0, 2, and 6 post-LPS infusion with an 18-gauge Vacutainer needle (BD, Franklin Lakes, NJ) and a 10 ml Vacutainer tube containing 158 USP Units of Sodium Heparin (BD). Tubes were inverted several times to ensure adequate mixing of the anti-coagulant and stored on ice for approximately 2 h before centrifugation at 800 x *g* for 20 min at 4°C. Plasma was then collected and stored at -20°C for future analysis.

4.3.3 LPS challenge

Each calf was fitted with a jugular catheter for the LPS challenge to allow for easier administration of the LPS solution and blood sampling. Twenty-four hours prior to the catheterization, a 50 cm section of polyethylene tubing (I.D. 1.19mm, O.D. 1.70mm; Intramedic, BD) was cut per calf and sanitized in a Nolvasan S solution (Pfizer, NY, NY). The day of catheterization each calf was secured in a headlock and the fur was clipped on top of the jugular vein and the area was cleansed with 70% ethanol. A 12-gauge, 1.5-inch metal hub needle (Hamilton, Reno, NV) was then inserted into the jugular vein, and 20-25 cm of the polyethylene tubing was inserted through the needle.

The needle was then removed, an 18-guage luer stub adapter (Intramedic) attached, and the catheter filled with heparinized saline. The following day, after morning feeding, a single dose of 0.25 ug of LPS/kg of body weight isolated from *E. coli* 0111:B4 (Sigma) was infused into each calf at hour 0. Heparinized blood samples were collected at -30 min, and then at hours 1, 2, 3, 5, and 7 post-infusion and stored on ice. Plasma was isolated within 2 h and stored at -20°C for future analysis.

4.3.4 Plasma IL-6 and TNF-α quantification

Concentrations of IL-6 in plasma samples were determined with a commercially available bovine IL-6 ELISA kit (Thermo Scientific, Rockford, IL). The capture and detection antibodies were both plated at 1:100 and the streptavidin-horseradish peroxidase was plated at 1:400. Recombinant bovine IL-6 (Thermo Scientific) was used at the assay standard. Concentrations of tumor necrosis factor alpha (TNF-α) were determined via RIA as previously described (Elsasser et al., 2005).

4.3.5 Dermal fibroblast isolation

Skin biopsies were collected from the ELL (n=5) and ELS (n=5) calves as previously described (Kandasamy et al., 2011). Briefly, dermal fibroblasts (DF) were isolated following a 6 h digest with 0.5% collagenase type I enzyme (Life Technologies, Grand Island, NY). The collagenase-digested tissue was filtered, centrifuged, and the cell pellet was reconstituted with Dulbecco's modified Eagle medium (DMEM; Hyclone Laboratories, Logan, UT) containing 10% fetal bovine serum (FBS; Hyclone Laboratories), a 1X antibiotic cocktail (100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B; Hyclone Laboratories), and 1X insulin-

transferrin-selenium (ITS; Mediatech, Herndon, VA) and seeded into a 25-cm^2 flask (Corning Inc., Corning, NY). Media was replaced after an initial 16 h incubation with fresh DMEM (10% FBS, 1X antibiotic cocktail, and 1X ITS). Once confluency was reached, cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded into a 75-cm^2 flask. After approximately 4 days, cells were expanded into three 75-cm^2 flasks. Finally, confluent cultures were lifted with trypsin and aliquots of the third passage were diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma) and cryopreserved in liquid nitrogen for subsequent challenges.

4.3.6 Monocyte isolation and macrophage culturing

A 180 ml blood sample was obtained from each of the ELL (n=5) and ELS (n=5) calves via jugular venipuncture with a 60 cc syringe pretreated with heparin (10,000 USP units/ml; Sagent Pharmaceuticals, Schaumburg, IL) and immediately transferred to heparinized vacutainer tubes (BD). Samples were stored on ice for approximately one hour prior to processing. Vacutainer tubes were centrifuged at 800 x *g* for 20 min at 4°C. Peripheral blood mononuclear cells (PBMC) were removed and treated with red blood cell lysis buffer (Biolegend, San Diego, CA). No more than five buffy coats were treated with 7.5 ml of 1X red blood cell lysis buffer and inverted for 30 s. Two washes were then performed using sterile Dulbecco's phosphate-buffered-saline (DPBS, Hyclone Laboratories) by centrifuging samples at 500x *g* for 5 min at room temperature. After the second wash, cells were resuspended in 30 ml of DPBS and 10 ml of a Percoll (GE Healthcare, Sweden) solution with a specific density of 1.077 g/cm^3 (mixture with 1.5 M NaCl and 30% endotoxin-free water) was carefully underlain below

the sample. The Percoll gradient was centrifuged at 400 x *g* for 40 min, at room temperature, with no brake. The PBMC layer was removed from the interface of the DPBS and Percoll and placed into a clean polypropylene tube. One more DPBS wash was performed as stated above to remove residual Percoll. After washing, PBMC were resuspended in 30 ml DPBS and centrifuged at 250 x *g* for 12 min at room temperature to remove platelets. After the platelet spin, the cells were resuspended in DMEM, 15% FBS, 1X ITS and 1X penicillin-streptomycin (100 U/ml of penicillin and 100 µg/ml of streptomycin). The PBMC were then transferred to a 25-cm^2 flask and incubated in a humidified chamber at 37°C and 5% CO_2 for 3 h. Non-adherent cells were removed and placed in a clean polypropylene tube and adherent cells were washed with DPBS and fresh DMEM media was added. Non-adherent cells were transferred to a second 25-cm^2 flask and those that had adhered overnight were washed the next morning and fresh media was added. Monocytes were incubated for 5 d and media was replaced as needed. After 5 d, cells were lifted with 0.25% trypsin, and the 3 h cultures and overnight cultures were combined and transferred to a 12-well plate at a concentration of 1.0 x 10^5 cells/ml in a total volume of 1 ml. The cells were cultured for 3 more days to allow for complete macrophage differentiation.

4.3.7 In vitro challenges

Aliquots of fibroblast cultures isolated from the ten bull calves were revived in parallel and grown to confluency in a 75-cm^2 flask. Cells were then detached with trypsin, counted, and seeded into 6-well plates at a concentration of 1.25 x 10^5 cells/ml in 2 ml total/well. Following a 24 h incubation, cells were exposed to 500 ng/ml of ultra-

pure LPS isolated from *E. coli* 0111:B4 (Sigma) or fresh culture media for 24 h. Media was then collected and centrifuged at 10,000x *g* for 1 minute to remove cell debris and the supernatant was stored at -20°C for future analysis. Cells were lysed at hours 0 and 24 post-LPS, and RNA was extracted at stored at -80°C (5 Prime, Gaithersburg, MD) until future analysis.

After 8 days in culture, macrophages were treated with either media or media containing 100 ng/ml of LPS isolated from *E. coli* O111:B4 for 24 h, after which, media was removed and centrifuged at 10,000x *g* for 1 min and supernatant was stored at -20°C until further analysis. Cells were lysed at hours 0 and 24 post-LPS, and RNA was extracted at stored at -80°C (5 Prime, Gaithersburg, MD) until future analysis.

4.3.8 Quantification of in vitro immune response proteins

Levels of IL-8 protein produced from fibroblast and MDM cultures following an LPS challenge were determined in duplicate with a commercially available bovine IL-8 ELISA kit (Mabtech, Cincinnati, OH) as per manufacturer's instructions with some modifications. A solution of 1 μg/ml of the monoclonal coating antibody (MT8H6) was plated in 0.05 M bi-carbonate buffer on a high affinity 96-well ELISA plate (Corning Life Sciences, Tewksbury, MA) and incubated overnight at 4°C. Media samples or recombinant bovine IL-8 standard (Thermo Scientific) were plated following a series of three washes with a solution of DPBS and 0.05% Tween-20 (DPBS-T; Fisher Bioreagents, Fair Lawn, NJ) and incubated for 2 h at 20°C. Plates were washed with DPBS-T and a monoclonal detection antibody (26ES-Biotin) was plated at 0.025 μg/ml and incubated for 1 h at 20°C. After a series of washes, streptavidin-horseradish

peroxidase (Sigma) was plated at 0.1 μg/ml and incubated for 1 h. Lastly, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Fisher Scientific, Pittsburgh, PA) was added for approximately 7 min, and the reaction was stopped with 1M H_2SO_4.

Concentrations of TNF-α protein in macrophage-conditioned media were determined in duplicate using a custom sandwich ELISA as described above except: coating antibody (polyclonal rabbit anti-bovine TNFα, 1 mg/ml, Thermo Scientific) was diluted 1:200 in 0.05 M bi-carbonate buffer, recombinant bovine TNF-α (Kingfisher Biotech, Inc., St. Paul, MN) was used as standard, and biotinylated secondary antibody (rabbit anti-bovine TNF-α, Thermo Scientific) was diluted 1:1,000 to a concentration of 0.25 μg/ml in DPBS-T. Detection limit of the assay was 156 pg/ml. The concentrations of IL-1β were similarly quantified using a commercially available sandwich ELISA (Thermo Scientific). The detection limit of the assay was 62 pg/ml. All ELISA reactions were quantitated by measuring absorbance at 450nm and analyzed by a 4-parameter analysis with optical density corrected against blank wells (Synergy-HT, Bio-Tek, Winnoski, VT).

4.3.9 Quantitative real time-PCR

Dermal fibroblasts (DF) and monocyte-derived macrophages (MDM) were isolated and cultured for an LPS challenge as described above for each of the ten calves and cell lysate was collected at hours 0 and 24 post-LPS for each culture. The PurefectPure RNA Cultured Cell extraction kit (5 Prime), which includes a DNase treatment step to eliminate DNA contamination, was used to extract total RNA from the cell lysate. RNA concentration was determined using a Qubit Spectrofluorometer (Life

Technologies, Carlsbad, CA). The Improm II Reverse Transcriptase Kit (Promega, Madison, WI) was utilized to complete first strand cDNA synthesis. The following genes were selected for real-time PCR analysis: *TLR4, IL-6, IL-8* (in both DF and MDM cultures) and *CD14, IL-1β receptor*, *and IL-1β* (in MDM cultures only). Expression levels of these genes were determined by quantitative real-time PCR (qRT-PCR) with a 7500 Fastrun Machine (Applied Biosystems, Carlsbad, CA) using Fermentas Maxima SYBR Green/Fluorescein qPCR Mastermix (Thermo Scientific): An endogenous control gene, *β-actin* was used for normalization of the target genes. Sequences for the constructed primers are listed in Table 1.

4.3.10 Statistical analysis

Differences in rectal temperatures, as well as concentrations of plasma TNF-α and IL-6 were determined between the ELL and the ELS calves following the early life treatment and the LPS challenge by a repeated measures ANOVA using Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Differences in immune response proteins and gene expression following an LPS challenge between the fibroblast and monocyte-derived macrophage cultures collected from the ELL and ELS calves were determined by an unpaired t-test in Prism 6.0.

4.4 Results

Blood samples were collected from each calf at hours 0, 2, and 6 following the early life treatment (LPS or saline) to determine concentrations of two key inflammatory cytokines, IL-6 and TNF-α. Prior to the treatment, levels of IL-6 (Figure 2A) and TNF-α

(Figure 2B) were similar between the ELL and ELS calves. However, by hour 2 post-treatment levels of IL-6 and TNF-α in the ELL calves had reached peak values ($P < 0.05$; 14.4 ± 3.1 and 3.2 ± 1.0 ng/ml, respectively) while no increase in either cytokine was observed in the ELS calves. At 6 h post-treatment, IL-6 levels remained elevated ($P < 0.05$) in the ELL calves and plasma TNF-α had decreased to pre-treatment levels, with no detectable changes in plasma IL-6 or TNF-α from the ELS calves. Moderate respiratory distress, and lethargy was typically observed in the ELL animals. Rectal temperatures did not increase due to the early life treatment in either group and were similar between the ELL and the ELS calves (Figure 2C).

Twenty-five days after the early life treatment, an LPS challenge was performed on all ten calves to compare the LPS response between calves that had received LPS (n=5) and calves that had received saline (n=5) in early life. Following an LPS bolus of 0.25 μg/kg of body weight, plasma levels of IL-6 increased in both the ELL and ELS calves, reaching similar peak values (6.7 ± 0.6 and 6.1 ± 0.7 ng/ml) at hour 3, and decreasing slightly by hour 7 post-infusion (Figure 3A). Plasma TNF-α reached maximum concentrations in ELL calves 1 h following LPS (4.8 ± 1.3 ng/ml) while TNF-α concentrations peaked in ELS calves 2 h post-LPS (3.9 ± 1.1 ng/ml). Despite the early temporal differences, TNF-α levels were similar between ELL and ELS calves for the remainder of the challenge (Figure 3B). Rectal temperatures following the LPS challenge decreased slightly in both groups, as shown in Figure 3C, however, there were no differences observed between the ELL and ELS calves.

Skin biopsies were collected from each calf 13 days after the early life treatment and 12 days before the LPS challenge to isolate dermal fibroblasts (DF). This time point

was selected in anticipation that any epigenetic modifications resulting from the early life LPS treatment that could influence the fibroblast LPS response would be present in our cellular model. Aliquots of fibroblasts from each animal were revived and challenged in parallel with LPS (500 ng/ml) for 24 h, and levels of IL-6 and IL-8 protein secreted in media were used as indicators of that fibroblast culture's LPS response. Fibroblasts isolated from the ELL and ELS calves at approximately 20 d of age produced similar levels of IL-6 (Figure 4A) and IL-8 (Figure 4C) protein following LPS exposure.

Monocyte-derived macrophages (MDM) were isolated from each of the calves (n=10) 21 days after the early life treatment and 4 days before the LPS challenge to determine if the in vitro LPS response from the MDM would be dampened due to the early life LPS treatment. MDM cultures were seeded into 6-well plates, incubated for 24 h, and then challenged with LPS (100 ng/ml) for 24 h. Concentrations of IL-6 and IL-8 protein produced by the MDM cultures following LPS exposure were similar between the treatment groups (Figure 4B and 4D). Additionally, levels of IL-1β and TNF-α were determined in MDM supernatant following LPS treatment (Figure 4E and 4F, respectively). Each of the immune response proteins measured in MDM cultures established from the calves were similar between the two early life treatment groups (ELL vs. ELS).

Expression levels of genes involved with the LPS recognition and response pathway were investigated in both the DF and MDM cultures established from all calves. Total RNA was collected from DF and MDM at hours 0 and 24 post-LPS and gene message was determined by qRT-PCR. Expression of *TLR4*, the receptor responsible for LPS recognition, was consistent following LPS stimulation and was not different between

the ELL and ELS groups in either DF or MDM cultures, but was much greater in the MDM cultures (Figure 5A and D). The cytokine *IL-6* and chemokine *IL-8* are critical immune response genes following LPS exposure, and the expression of these genes increased from hour 0 to hour 24 in both cellular models (Figure 5B and E). However, no differences were observed between the ELL and ELS calves in either cellular model, although the expression of both IL-6 and IL-8 was much greater in the MDM cultures. Levels of expression of three other genes (*CD14*, *IL-1β receptor*, and *IL-1β)* were determined in control and LPS-treated MDM cultures from the ELL and ELS calves (Figure 5G, H, and I). The expression of *IL-1β* was markedly induced by LPS treatment of the MDM cultures, while the expression of *CD14* and the *IL-1β receptor* were not affected. However, there were no differences in gene expression between the cultures established from the ELL and ELS calves.

4.5 Discussion

Previous work from our laboratory and others has shown that dairy cattle manifest a wide range of immune responses following experimentally induced *E. coli* (Kornalijnslijper et al., 2003, Bannerman et al., 2004, Kanasamy et al., 2011) or *Staphylococcus aureus* (Schukken et al., 1999, Bannerman et al., 2004, Benjamin et al., 2015) mastitis. Some animals exhibit a much more robust inflammatory response during mastitis, characterized by high levels of IL-8, IL-6, and TNF-α, which can lead to severe clinical symptoms and damage to the mammary tissue. Inter-animal variation in the expression of genes within the LPS response pathway, including *TLR4*, the receptor for LPS, may lead to individual differences in the ability to recognize and respond to gram-

negative pathogens (Kandasamy and Kerr, 2012). A genetic component to individual differences is highly likely, but new research suggests that epigenetics may also play a role. For example, we have found that the LPS responsiveness of an animal's fibroblasts changes with age. In side-by-side cultures of cells, recovered from cryopreservation but originally collected from the same animals at different ages, we find a marked increase in response to LPS stimulation. Furthermore, the age-dependent difference in LPS response of these cells can be abolished by treatment with epigenetic modifiers (Green and Kerr, 2014). Expanding on the inter-animal variation observed in the fibroblast model, cultures established from dairy and beef animals displayed contrasting responses following an in vitro LPS exposure. Whole transcriptome analysis of LPS-treated cultures revealed large breed-dependent differences in the expression of many LPS-responsive genes, including *TLR4*, *IL-8*, *TNF-α*, *CCL5,* and *CCL20* (Benjamin et al., 2016). These genes, which are associated with the inflammatory response to LPS, were expressed between 2.5- and 7.0-fold higher in Holstein fibroblast cultures as compared to Angus cultures. While genetics may contribute to the contrasting in vitro LPS responses between these breeds, offspring from each breed experience very different early life environments, which could lead to varying degrees of epigenetic modifications between the dairy and beef breeds, potentially moderating inflammatory gene expression.

4.5.1 Impact of neonatal inflammation

Several studies have shown that inflammation in the neonatal period (shortly after birth) can lead to sustained suppression of the innate immune response to a subsequent inflammatory challenge. Ellis et al. (2005) observed reduced plasma levels of TNF-α and

IL-1β as well as a suppressed febrile response following an LPS challenge of adult rats that had been exposed to LPS at 14 days of age compared to saline-exposed controls. Similarly, adult rats that had previously received a neonatal dose of LPS (100 ug/kg) at 14 or 21 days of age produced a lower febrile response, compared to neonatal saline controls, during a subsequent LPS (50 ug/kg) challenge (Spencer et al., 2006). However, the timing of the neonatal exposure was found to be critical in that LPS treatments given at 7 or 28 days of age were not effective. Additionally, the suppressive effect is not observed if the adult challenge dose of LPS is increased to a septic dose of 1 mg/kg (Spencer et al., 2010). In the current study, we hypothesized that an early life LPS exposure would generate a low-responding phenotype in Holstein calves towards a subsequent LPS challenge. Surprisingly, ELL calves mounted a similar inflammatory response as the ELS calves during the later LPS challenge that was completed on all calves 25 days after the first LPS or saline exposure. Plasma levels of TNF-α and IL-6 during the later LPS challenge were comparable between treatment groups, showing the early life treatment with LPS (0.5 ug/kg) at 7 days of age did not dampen the subsequent inflammatory response to LPS (0.25 ug/kg). The doses used did produce substantial inflammation and may have been too high to generate the expected result.

Although other studies have shown that administering LPS in the neonatal period does reduce the LPS response in the adult, most of these studies have been completed in rats (Spencer et al., 2011, Wang et al., 2011). Different rates of development and maturity within the immune system between calves and rodents may be one explanation for the lack of differences between our LPS- and saline- treated calves. Offspring from species with a comparatively short gestation period, such as rats (length of 21 days), are born

with a less mature immune system than offspring from species with a longer gestation, cattle for example (length of 9 months) (Holsapple et al., 2003). A less mature immune system during the neonatal period in a rat pup may provide an opportunity for inflammation to epigenetically modify the genes of the immune response in the adult, whereas the immune system of a neonatal calf may be less vulnerable to those types of modifications during that same stage of life. Following parturition, the humoral and cell-mediated immune response of the calf is much lower than an adult cow, but within the first month of life, B-cell numbers increase and the cell-mediated response is similar to that of an adult animal (Barrington and Parish, 2001). The immune system in newborn rats is immature, and continues to develop during the postnatal period and although an immune response can be mounted at 21 days of age, it is not of the same magnitude as an adult animal (Holsapple et al., 2003). A comparison of the degree of immune system development of the rat pups in studies mentioned previously and the calves in this current study suggests the timing of the LPS exposure in the neonatal calves may have been too late to obtain a reduction in the inflammatory response towards an LPS challenge.

4.5.2 Fetal programming: maternal inflammation and nutrition

Exposure to bacterial molecules or inflammation while in utero can also alter aspects of an offspring's behavior, health, and development. Increases in anxiety and depression-like behaviors as well as an increased propensity towards developing schizophrenia have been observed in rodents whose dams suffered stress or inflammation during gestation (Ashdown et al., 2006, Depino, 2015). Additionally, several studies have shown that the inflammatory response of the offspring can be suppressed following in

utero LPS exposure. For example, Lasala and Zhou (2007) found that an intraperitoneal injection of LPS given to pregnant rats on day 18 of pregnancy greatly reduced the inflammatory response (serum levels of TNF-α, IL-1β, and IL-6) of the resulting pups when they were challenged with LPS at 21 days of age. In a similar study, adult rats that were exposed to LPS in utero at days 16, 18, and 20 of gestation have a blunted inflammatory response towards an LPS challenge as compared to saline-exposed rats (Hodyl et al., 2007). Similarly, offspring of mouse dams that were administered LPS approximately 12 h after fertilization had a diminished cytokine response following an LPS challenge at 40 weeks of age (Williams et al., 2011). Perhaps an in utero exposure of LPS, or maternally derived inflammatory cytokines, would cause a reduction in the inflammatory response towards a later LPS challenge in dairy calves. However, careful consideration of the timing and dosing of the immune stimulant for such a study must be taken, as previous studies conducted in dairy cattle have shown that experimental uterine bacterial infections and LPS infusions can cause abortions prior to gestational term (Miller et al., 1983, Giri et al., 1990).

4.5.3 LPS tolerance

Endotoxin tolerance is a protective mechanism that allows for innate immune cells to experience a transient unresponsive period of time following exposure to low levels of LPS (Biswas and Lopez-Collazo, 2009). Depending on the species, this refractory period can vary in duration, from roughly a week in rats (West and Heagy, 2002),10 days in dairy cattle (Petzl et al., 2012), to at least two weeks in human patients suffering from endotoxemia (Kox et al., 2011). Several studies have shown that a primary

result of endotoxin tolerance is a reduction or complete abolishment of TNF-α transcript and protein following a second exposure to LPS (West and Heagy, 2002). Within our study, the calves that were treated with LPS in early life were likely outside the range of a tolerant state during the subsequent LPS challenge that was given 25 days later. The levels of plasma TNF-α in these calves were actually higher during the later LPS challenge compared to levels reached during the LPS treatment in early life (4 ng/ml vs. 3 ng/ml, respectively). This demonstrates that the cells primarily responsible for executing LPS tolerance, monocytes and macrophages (Kox et al., 2011), were able to mount an unabated inflammatory response towards the later LPS challenge.

4.5.4 Cellular models of innate immunity

Extensive use of in vitro models to study the bovine innate immune response is evident, with several different cell types, including mammary epithelial cells (Pareek et al., 2005, Gilbert et al., 2013), neutrophils (Sohn et al., 2007, Revelo and Waldron, 2012), and whole blood (Ballou et al., 2015, Jahan et al., 2015) being utilized. However, each of these models has drawbacks, including invasive collection procedures, inability to cryopreserve for subsequent challenges, and a heterogeneous cell population. Conversely, monocyte-derived macrophages do provide a classical innate immune cell model that is relatively easy to collect and culture. Bovine monocyte-derived macrophages have been shown to be responsive to challenges with *Mycobacterium bovis* (Magee et al., 2012) and LPS (Taraktsoglou et al., 2011), resulting in the induction of many immune -response genes such as *IL-8*, *CCL5*, and *TLR4*.

In the current study, the establishment of fibroblast and monocyte cultures from each animal provided two cellular models to determine if the early life LPS induced epigenetic modifications within innate immune response genes that would be reflected in the in vitro LPS-response. Additionally, collection of these two cell types from each animal allowed for a comparison of the LPS response of two cellular models from the same animal. Fibroblasts are long-lived cells that are capable of modulating the inflammatory response as they express innate immune receptors such as TLRs and secrete various cytokines and chemokines following pathogen recognition (Buckley et al., 2001, Kandasamy et al., 2011, Benjamin et al., 2015). Alternatively, circulating monocytes are released from bone marrow, and depending on the environmental conditions, undergo apoptosis within a few days or migrate into tissues (Parihar et al., 2010). Once inside the tissue, monocytes will differentiate into macrophages that aid in the clearance of a pathogen or damaged cells (Parihar et al., 2010). These tissue-resident macrophages can survive for days to months depending on the needs of the host. Within this study, the main objective in collecting both monocytes and fibroblasts from the saline- and LPS-treated calves was to observe if the early life LPS created a change, either permanent or temporary, in the in vitro LPS response between the early life treatment groups. Monocytes are continually produced in the bone marrow by myeloid progenitor cells and a systemic cytokine response following LPS exposure may reach these stem cells and induce permanent epigenetic DNA modifications that could be detected in monocytes collected three weeks later. Dermal fibroblasts however are a more stable cell type than monocytes, but may still be susceptible to epigenetic modifications following exposure to a systemic inflammatory response. While epigenetic modifications

could be induced within fibroblast progenitor cells, it is more likely that the fibroblast cells we collected three weeks post-challenge were the same cells present at the time of the early life LPS treatment. This may create more transient epigenetic modifications, such as histone modifications, that are still capable of influencing the inflammatory response, but are potentially too short-lived to detect the effect after several rounds of cell division while in cell culture. While progenitor fibroblast cells could be affected, they proliferate at a slower rate and this effect may not be evident only three weeks post early life treatment. Surprisingly, the in vitro response following an LPS challenge on each of these cell types revealed similar profiles in both cytokine secretion and gene expression between the LPS- and saline-treated calves, which confirmed the lack of differences observed in vivo during the second LPS challenge. Interestingly, the monocyte-derived macrophages exhibited a much more robust IL-6 response following LPS stimulation as compared to the fibroblasts (Figure 4). Other groups have observed a similar induction of IL-6 in macrophages following LPS stimulation (Jian et al., 1995, Taraktsoglou et al., 2011) but, there have been few studies examining differences in the IL-6 response following LPS stimulation between bovine MDM and DF cultures. A comparison between mouse gingival fibroblasts and macrophages revealed that the mouse alveolar macrophage cell line produced less IL-6 following stimulation with LPS compared to the fibroblast cultures (Jones et al., 2010). While this is inconsistent with our findings, Jones et al. (2010) utilized a commercially available cell line from mice and challenged with a LPS dose of 1 μg/ml for 24 h. Our MDM model is derived from primary bovine monocytes that are allowed to differentiate into macrophages in culture, and we exposed

the cells to a lower LPS dose (100 ng/ml) for 24 h. Additional challenges that compare the LPS response of primary bovine MDM and DF may be of interest.

4.6 Conclusions

Variation in the innate immune response towards mammary infections exists in dairy cattle, with some animals exhibiting a mild inflammatory response and others suffering a severe inflammatory reaction that results in collateral damage or death. It has been shown that exposure to inflammation or stress either during early life or in utero can cause epigenetic modifications to the animal's innate immune response. The potential for this type of exposure to create a low responding phenotype in the offspring, one that mounts an effective inflammatory response without inflicting damage on host's tissue, is exciting. However, based on the results from this study, an early life exposure to LPS does not cause a dairy calf to mount a milder inflammatory response to a subsequent LPS challenge 25 days later compared to saline-treated control calves. Through the use of monocyte-derived macrophages and dermal fibroblasts, the lack of difference in vivo between LPS-treated and saline-treated calves was confirmed in vitro.

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Table 1 Oligonucleotide primers for expression quantification with real-time PCR.

(The top and bottom sequences are the forward and reverse, respectively)

Gene Symbol	Primer (5' to 3')	Reference
TLR4	ACTGCAGCTTCAACCGTATC TAAAGGCTCTGCACACATCA	Ibeagha -Awemu et al. (2008)
IL-6	TGAGGGAAATCAGGAAAATGT CAGTGTTTGTGGCTGGAGTG	Pareek et al. (2005)
IL-8	GCTGGCTGTTGCTCTCTTG AGGTGTGGAATGTGTTTTTATG	Pareek et al. (2005)
CD14	CTCCAGCACCAAAATGAC TCCTCTTCCCTCTCTTCC	Sohn et al. (2007)
IL-1βR	GCTCGTGTCCTCTCATCACA ACCTTTGTGCTGGTGAATCC	Mills et al. (2009)
IL-1β	CTCTCACAGGAAATGAACCGAG GCTGCAGGGTGGGCGTATCACC	Bougarn et al. (2011)
B-actin	GCAAATGCTTCTAGGCGGACT CAATCTCATCTCGTTTTCTGCG	Pareek et al. (2005)

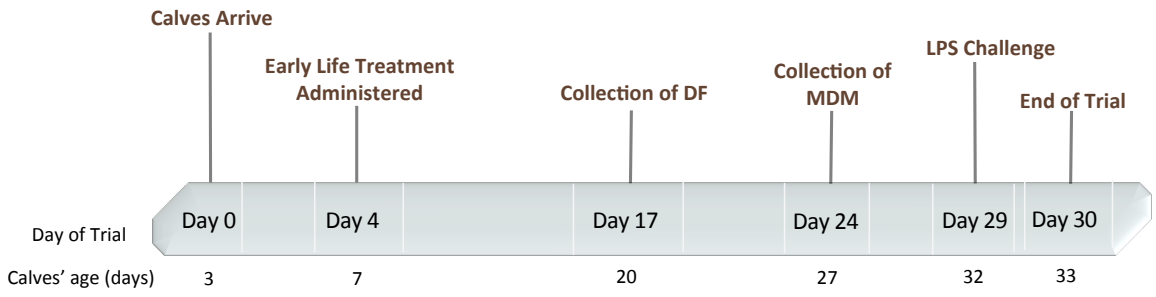

Figure 1 Timeline of the Study.

The effects of an early life LPS exposure on response to a subsequent LPS challenge were determined in ten neonatal Holstein calves. Trial days are shown in the gray bar, with the calf arrival date on day 0 and completion of the trial on day 30, at which point calves were sold. The average age of the calves and key events within the trial are denoted below and above the gray bar, respectively. Early life treatment at day 4 consisted of either an LPS or saline intravenous injection (n=5/treatment). Skin biopsies and blood samples were collected from each calf to isolate dermal fibroblasts (DF) and monocyte-derived macrophages (MDM), respectively. An LPS challenge was completed on all calves at day 29 of the trial to determine the affect of the early life treatment on response to the subsequent LPS challenge.

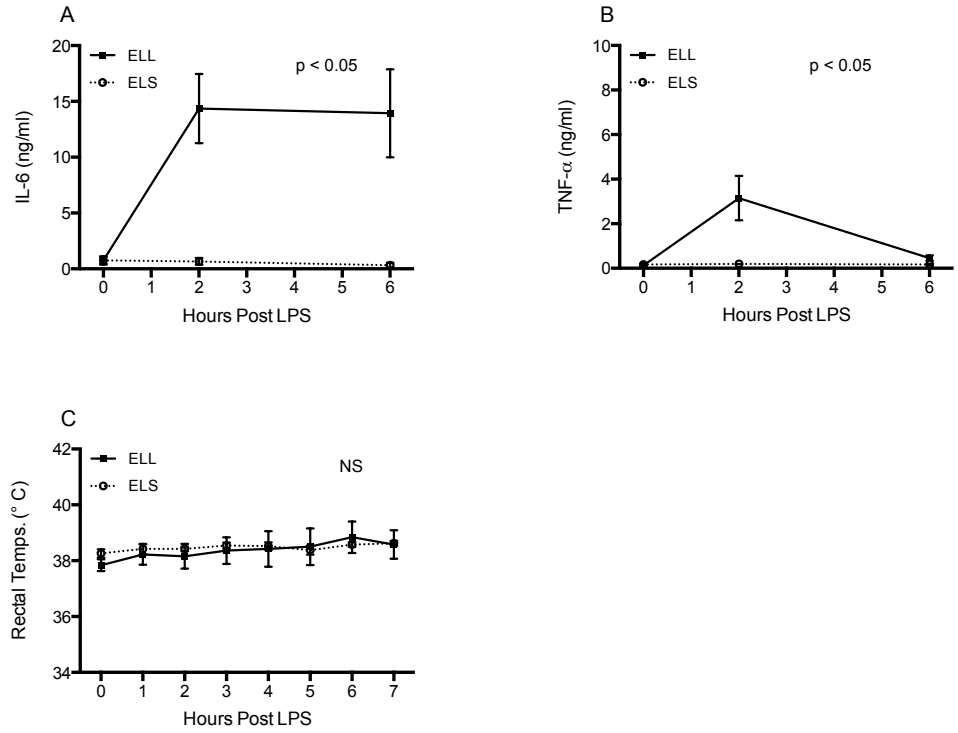

Figure 2 Plasma cytokine responses and rectal temperatures following the early life treatment.

Calves were split into two groups, one of which received LPS and the other received saline at 7 days of age. Blood samples were collected from each calf at hours 0, 2, and 6 following administration of the early life treatment. Plasma levels of IL-6 (A) and TNF- α (B) were determined after the early life treatment, and rectal temperatures were taken on animals from both groups (C). ELL= early life LPS and ELS= early life saline. Values are mean ± SEM.

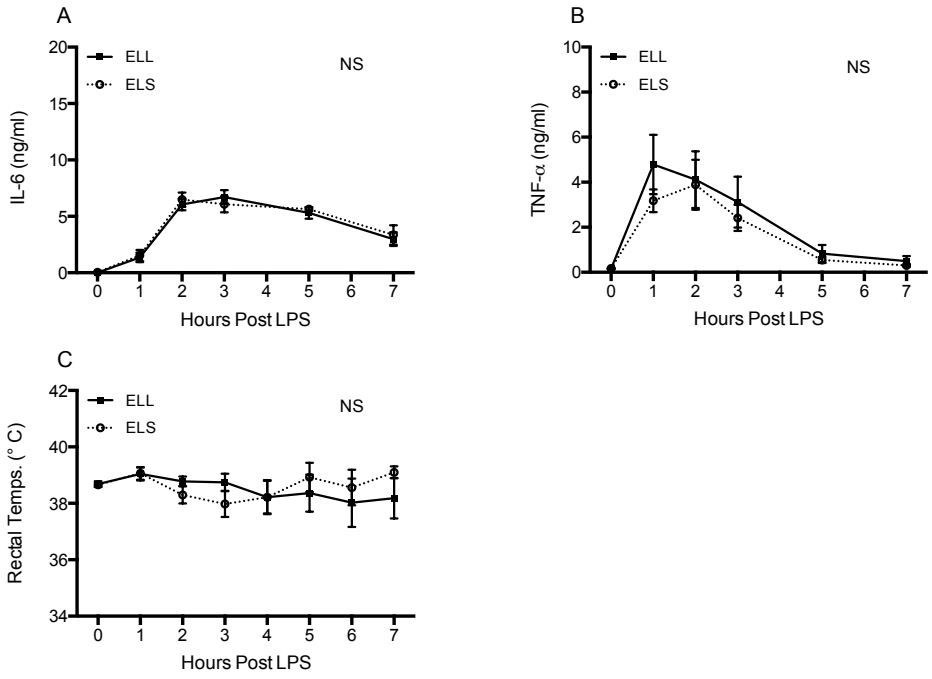

Figure 3 Plasma cytokine response and rectal temperature profiles following an LPS challenge.

Holstein bull calves were divided into two groups (n=5/group) based on an early life treatment consisting of either LPS (ELL) or saline (ELS) that was given at 7 days of age. A subsequent LPS challenge was completed on all calves at 32 days of age to determine if the early life treatment had an affect on the systemic response to the later LPS. Plasma concentrations of IL-6 (A) and TNF- α (B) were measured in the calves from the ELL and ELS groups following the LPS challenge. Rectal temperatures (C) of calves following the LPS intravenous infusion at 32 days of age were not different between the treatment groups. Values are mean ± SEM.

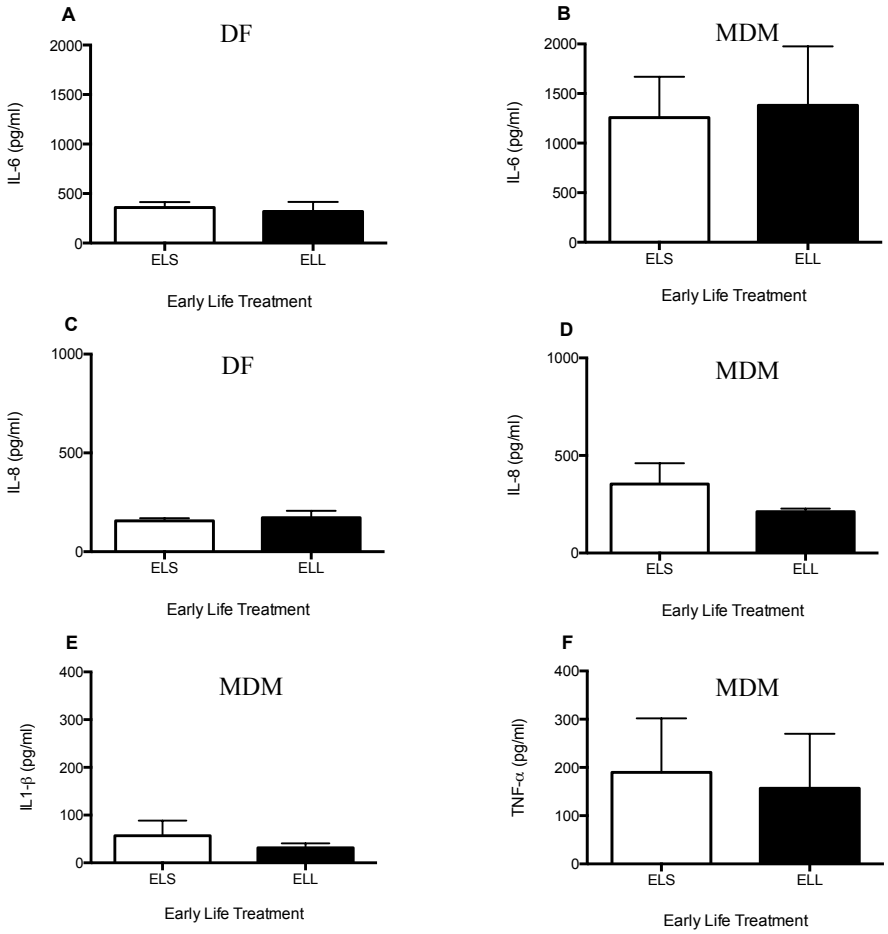

Figure 4 Effect of early life LPS on cellular response to LPS in vitro.

Dermal fibroblasts (DF) and monocyte-derived macrophages (MDM) were isolated from each calf (n=10) to determine if an in vivo LPS exposure during early life would influence the response of these cell types to an in vitro LPS challenge. A 24 h LPS challenge was completed on each cell type, however DF were treated with 500 ng/ml of LPS and MDM were treated with 100 ng/ml of LPS. The amount of secreted IL-6 from DF (A) and MDM (B), IL-8 from DF (C) and MDM (D) following LPS exposure was determined via an ELISA. Panel (E) and (F) represent the levels of IL-1β and TNF-α secreted by MDM post-LPS (concentrations of IL-1β and TNF-α were undetectable in LPS-treated fibroblast cultures). ELL= early life LPS, ELS= early life saline. There were no significant differences between treatment groups. Values are mean ± SEM.

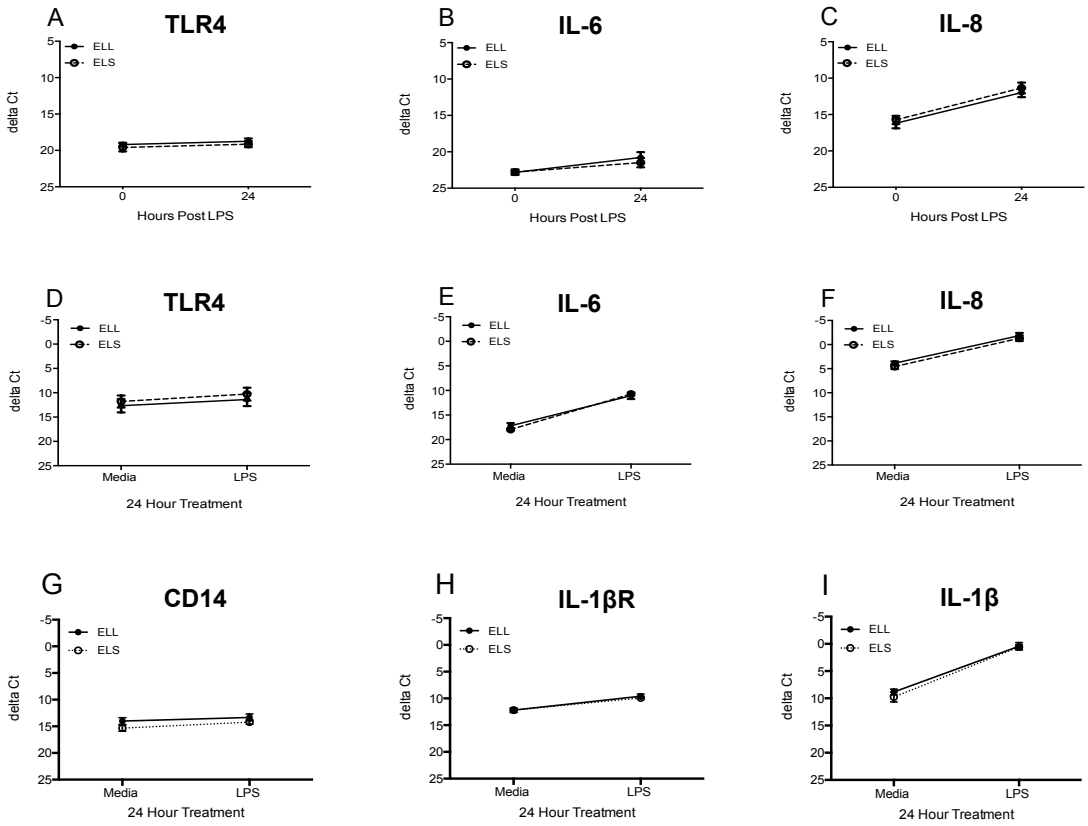

Figure 5 Expression levels of LPS recognition and response genes.

Dermal fibroblasts (DF) from each calf were revived and challenged with 500 ng/ml of LPS for 24 h and total RNA was collected at hours 0 and 24. A similar challenge was completed on monocyte-derived macrophages (MDM) isolated from each calf; however, an LPS dose of 100 ng/ml was used. Gene expression levels of TLR4, IL-6, and IL-8 post-LPS were investigated on both DF and MDM cultures using qRT-PCR. Expression levels from DF cultures are presented in panels (A) TLR4, (B) IL-6, and (C) IL-8, while expression levels of MDM cultures are in panels (D) TLR4, (E) IL-6, and (F) IL-8. Panels (G), (H), and (I) represent the expression levels of CD14, IL-1 β receptor, and IL-1 β from MDM, respectively. Expression values for these three genes were not determined in DF cultures. ELL= early life LPS, ELS= early life saline. There were no significant differences between treatment groups. Values are mean ± SEM.

Chapter 5: General Conclusions

A common goal within the dairy industry is to selectively breed animals for increased resistance to mastitis. Although advancements in the control of mastitis have occurred through improved management strategies that can reduce an animal's exposure to pathogens, dairy cattle are still affected by mastitis. This can be partially attributed to the lack of an effective selection trait, leading to a low heritability of mastitis resistance in dairy animals. Currently, milk somatic cell count (SCC) is the trait used as an indication of an animal's ability to resist mastitis. The SCC value represents the number of cells present in the milk, and can be used as an indicator of the infection status of an animal. Although monthly SCC tests can provide a monitoring system for fluctuations in an animal's SCC over a lactation, some infections can occur and resolve quickly and may be missed by the SCC test. In these cases, the animal may incorrectly be classified as resistant to mastitis.

In addition, the wide range in the clinical responses associated with mastitis between animals can make it difficult to effectively select for a desirable phenotypic response. As the innate immune system is responsible for pathogen recognition, and both the initiation and the resolution of the inflammatory response following a mammary infection, the magnitude of an animal's innate immune response can influence the degree of inflammation and clinical symptoms associated with mastitis. Researchers have not been able to agree on what the ideal phenotype is following mastitis, as some have suggested that a rapid, robust response is more effective for *E. coli* clearance (Burvenich et al., 2003, Rinaldi et al., 2010, Quesnell et al., 2012), while others have shown that a more mild response is sufficient to clear *E. coli* mammary infections (Kandasamy et al., 2011, Petzl et al., 2012). A better understanding of what host factors could lead to the

variation in the innate immune response and how those variations effect an animal's ability to recover from mastitis may provide insight into which phenotype is a more desirable one for producers.

The current studies aimed to determine if a particular innate immune response phenotype had a greater propensity towards developing a chronic *S. aureus* mammary infection, as well as advancing the knowledge on what factors could be causing the inter-animal variation in the innate immune response. First, it was determined that the magnitude of the IL-8 response from dermal fibroblasts challenged *in vitro* with PAM2CSK4, a TLR2/6 agonist, was predictive of an animal's *in vivo* innate immune response towards a *S. aureus* intra-mammary challenge. Further *in vitro* expression analysis on these fibroblasts challenged with PAM2SCK4 revealed that high responding cultures had greater expression of TLR2 and 6, which recognize bacterial lipopeptides and synthetic TLR2 agonists. The higher expression of these two genes may allow for the increased binding of bacterial ligands, thus leading to greater activation of transcription factors and the expression of inflammatory mediators such as IL-8 and IL-6. During the intra-mammary challenge, animals predicted to have a robust innate immune response by the *in vitro* cell model secreted much higher levels of IL-8 into their milk, leading to a greater recruitment of neutrophils into the infected quarter. In addition, greater levels of bovine serum albumin (BSA) were present in the milk from the infected quarter of the high responders, indicating a greater breakdown of the blood-milk barrier and more tissue damage to the mammary gland. Infection rates throughout the challenge were similar between the phenotypes; however, one low responder did clear the infection early in the experiment and remained free of infection till the conclusion of the trial, while a second

low responder was culture negative between day 4 and 14 post-infection, upon which, the challenge strain was re-isolated from the challenged quarter. These observations suggest that the heightened inflammatory response generated by the high responders offered no advantage in bacterial clearance or in the ability of the animal to resist developing a chronic *S. aureus* infection, and instead, led to greater damage to mammary tissue and a more drastic decrease in milk quality.

In an effort to further elucidate the causes for the variation observed in our cohort of dairy cattle, we elected to compare the *in vitro* responses of fibroblasts isolated from Holstein and Angus animals. These two breeds of cattle have undergone extensive selection for different production traits, which may introduce genetic variants within key innate immune response genes. Furthermore, the early life environment of calves from these two breeds differ, thereby providing an opportunity for differences in epigenetic modifications between Holstein and Angus animals. A comparison of the fibroblast LPS response between these two breeds revealed that Holstein cultures produced more IL-8 following LPS stimulation. The observation that Holsteins responded more robustly than Angus is in agreement with several other groups that have shown the Holstein breed suffers more severe clinical symptoms following parasite challenges, and has greater expression of several pro-inflammatory genes at tick attachment sites compared to breeds of *B. indicus* cattle.

As these observed *in vitro* differences could be due to both genetic and epigenetic differences between the Holstein and Angus breed, the transcriptome of unstimulated and LPS-treated fibroblast cultures from both breeds were examined using RNA-Seq, and the DNA methylation patterns were compared on genomic DNA isolated

from unstimulated cultures with MIRA-Seq (Methylated CpG Island Recovery Assay). RNA-Seq analysis revealed a number of innate immune response genes, including TLR4, IL-8, CCL20, and TNF-α, were differentially expressed between Angus and Holstein cultures. As TLR4 expression was greater in Holstein cultures, this may lead to an increased ability to recognize and respond to LPS by this breed, and a greater capacity to bind LPS would allow for increased activation of the transcription factors that promote the expression of inflammatory mediators. Surprisingly, there were few differences in the DNA methylation levels. MIRA-Seq analysis did uncover 49 regions within the genome that had different rates of methylation between Holstein and Angus cultures, however, innate immune response genes were under-represented in these regions.

The breed difference in the innate immune response is exciting, and provides an opportunity to explore the effects that the early life environment can have on the magnitude of the inflammatory response in an animal. While beef calves suckle from the dam and have access to pasture and the rest of the beef herd, dairy calves are separated from the dam, fed a milk-replacer based diet, and often housed individually. Differences in the early life environment such as the rates of exposure to commensal and pathogenic bacteria between suckled calves and bottle-fed calves may induce epigenetic modifications in genes involved with the innate immune response. Our analysis of epigenetic differences between Holstein and Angus fibroblasts was done exclusively with MIRA-Seq, and while this technology provides a description of the genome-wide methylome, its sequencing resolution is approximately 100bp. Smaller changes in the DNA methylation pattern would have been missed in this analysis. Future studies comparing the DNA methylation levels between dairy and beef cattle using a more

sensitive technique, such as RRBS or whole genome bisulfite sequencing, are warranted. If an increase in methylation is identified as a key factor leading to a reduced innate immune response, perhaps feeding methyl donors to the dairy dam during pregnancy can induce greater methylation of inflammatory genes in the fetus, thereby creating a more desirable, low responding offspring.

Several studies completed in rodents have shown that events occurring in early life can have long-lasting effects on the innate immune response of the adult animal. Exposure to bacterial compounds such as LPS has been shown to induce a reduction in pro-inflammatory mediators following a subsequent LPS challenge in adulthood, essentially creating a low responding individual. As the research presented here has shown that a low responding phenotype is desirable in dairy animals, we attempted to replicate these results from the rodent studies in dairy calves. However, our efforts proved ineffective, as calves exposed to the early life LPS had similar levels of inflammatory mediators in serum following a subsequent LPS challenge as the calves that had received saline in early life.

This work on dairy calves was novel, therefore, the dosage and timing of the early life LPS was based off previous studies, which had been completed in rodents. Bovines are known to be more sensitive to LPS as compared to mice and rats, so a much lower dose of LPS was chosen in hopes of avoiding severe systemic responses, but still inducing a moderate degree of inflammation that might lead to epigenetic changes in innate immune response genes. However, this dose of LPS still produced a very vigorous response from the calves, with substantial inflammation and clinical symptoms. Perhaps an even lower dose would be better as this response may have been too high for the

desired result. Additionally, the timing of the LPS exposure in the calves may not have been at a critical time to induce epigenetic modifications of innate immune response genes, as the timing of the LPS in rodent studies was shown to be crucial for the induction of a lower innate immune response. Future studies examining the effects of different LPS doses or the timing of the early life exposure on the innate immune response of the calf are warranted.

As the world's population increases, the demand for food, and greater efficiency in the production of that food increases as well. Within the dairy industry, the increased production of milk to fill the demand for dairy products needs to be cost-effective for the producer while maintaining the welfare of the dairy cow. Animals that respond more vigorously to mastitis can make both of those tasks difficult, as these animals often require more extensive treatment, have greater losses in milk production, and generally suffer greater pain and discomfort than animals with a milder inflammatory response. These studies presented here have advanced the knowledge of the innate immune response of the bovine. We have shown that a reduced innate immune response is more beneficial for the dairy animal, as there are less pro-inflammatory mediators secreted into the milk and less tissue damage to the mammary gland. In addition, this milder response does not increase an animal's propensity towards the development of a chronic *S. aureus* mammary infection.

Furthermore, the examination of breed-dependent differences in the innate immune response resulted in the development of a panel of genes that show differential expression between low and high responding phenotypes. These biomarkers can be used in future *in vitro* studies to more accurately define an individual's phenotype. As we have

shown that a low innate immune response is more desirable in the dairy animal, identifying animals with this phenotype at a young age, or inducing that phenotype through environmental factors would be financially beneficial for the producer since heifer rearing costs are second to feed costs on a dairy farm. Our attempt to induce a lower innate immune response in dairy calves was not successful, however, this work provides a foundation on which future studies can build on in determining what environmental cues may result in the creation of a low responding dairy animal.

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Appendix A: LPS response of dermal fibroblasts isolated from genomically tested dairy cattle

A.1 Experimental Design and Rationale

The innate immune response can be quite variable between animals during a case of mastitis with some animals experiencing mild inflammation and others exhibiting a more severe inflammatory response. Selecting for an effective innate immune response that results in pathogen clearance while minimizing collateral damage to the mammary tissue is desirable by dairy producers. Genomic testing has been used within the dairy industry for animal selection since 2007 (Marcos V.B. Silva, 2014); and since it's implementation, it has allowed dairy producers to determine the genetic potential of offspring at a young age, thereby reducing the generation interval and increasing the accuracy of selection (Weigel et al., 2012). The most cost-effective way for a producer to estimate the genetic merit of an animal is with SNP chips; which contain probes for thousands of known SNPs in the bovine genome that have been linked (directly or in-directly) to different production, health, and conformation traits of the dairy cow (Zare et al., Kadri et al., 2015).

Productive life (PL) is a trait in which the assigned numerical value indicates the number of additional months an individual is expected to stay within the herd compared to the average Holstein animal that was genomically tested (Holstein Association USA); with the average Holstein bull stud having a PL of 0.95. An animal with a PL of 3.5 would be expected to have an additional 3.5 productive months in the herd compared to the average Holstein, and an animal with a PL value of -1.0 would be expected to have one less productive month than the average Holstein. In this sense, PL is considered a fitness trait, as it takes into consideration many phenotypes that contribute to the overall health and longevity of the animal, including feet and leg conformation, udder

attachment, milk production, and conception rates. Therefore, PL may be a useful indicator of an animal's ability to mount an effective innate immune response to mastitis without suffering severe clinical symptoms that can be accompanied by mammary tissue damage and potential removal from the herd. Using our fibroblast cellular model, we sought to determine if PL could be a predictor of an animal's in vitro innate immune response phenotype.

A.2 Materials and Methods

A.2.1 Experimental Design and Animal Selection

The owner of Welcome Stock Farm (Saratoga Springs, NY) was performing 50K SNP chip analysis (Illumina, Inc., San Diego, CA) on all of his animals, and agreed to give our laboratory access to the SNP chip data files. We selected two groups of adult female Holsteins: one with a high PL value (n=6; avg. PL of 5.4 ± 0.46) and one with a low PL value (n=5; avg. PL 0.36 ± 0.89) for inclusion on this study (Table 1). We did not have access to health records or monthly SCC of the animals selected for this study. The University of Vermont's Institutional Animal Care and Use Committee approved all animal procedures before commencement of the study.

A.2.2 Dermal Fibroblast Isolation

Dermal fibroblasts were isolated as described previously (Kandasamy et al., 2011). Briefly, skin biopsies were collected from each animal, and dermal fibroblasts were isolated following a 6 h digestion with collagenase type I (Life Technologies, Grand Island, NY). Cells were seeded into a 25-cm^2 flask (Corning Inc., Corning, NY) and cultured in Dulbecco's modified eagle medium (DMEM; Hyclone Laboratories, Logan,

UT) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories), 1X insulin-transferrin-selenium (ITS; Mediatech Inc., Herndon, VA), and 1X antibiotic cocktail (100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B; Hyclone Laboratories). Once confluent, cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded into a 75-cm^2 flask (Corning Inc.) for approximately 2 days, after which, cells were detached and expanded into three 75-cm^2 flasks. Following 3 days in culture, fibroblasts were detached and frozen in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma- Aldrich, St. Louis, MO) and aliquots were cryopreserved in liquid nitrogen for subsequent challenges.

A.2.3 Dermal Fibroblast Challenges

Aliquots of dermal fibroblasts were revived from cryopreservation and seeded into a 75-cm^2 flask for approximately 3 days to allow for adequate cell expansion. Once confluent, cells were lifted with trypsin and seeded into a 6-well plate (Corning Inc.) at a cell concentration of 1.25 x 10^5 cells/ml in 2 ml of media total and incubated for 24 h. After removal of old media, cells were treated with either fresh media, media containing 100ng/ml of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich), or media containing 1ng/ml of recombinant bovine IL-1β (AbD Serotech, Raleigh, NC) for 24 h. Following the challenge, media was collected from each well, centrifuged at 10,000 x *g* for 1 min to remove cellular debris and stored at −20°C until future analysis for IL-8 and IL-6 protein.

A.2.4 IL-8 and IL-6 ELISA

The concentration of IL-8 in conditioned media samples was quantified by a custom sandwich ELISA as previously described (Kandasamy et al., 2011). Mouse anti-bovine IL-8 (clone 170.13 from Samuel Maheswaren, University of Minnesota, St. Paul, MN) and biotinylated goat anti-human IL-8 (R&D Systems Inc., Minneapolis, MN) were used as capture and detection antibodies, respectively, and recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) as the assay standard. The concentration of IL-6 in conditioned media was determined with a commercially available bovine IL-6 ELISA kit (Thermo Scientific). The capture and detection antibodies were plated at 1:100 and the streptavidin-horseradish peroxidase was plated at 1:400. Recombinant bovine IL-6 was used as the assay standard.

A.2.5 Statistical Analysis

Differences in the levels of IL-8 and IL-6 between low and high PL cultures were determined using an unpaired t-test in Prism 6.0 (Graph Pad Prism 6.0).

A.3 Results

Fibroblasts isolated from animals with a high PL (n=6) and a low PL (n=5) were challenged for 24 h with either LPS (100ng/ml) or IL-1β (1ng/ml) and media was collected to determine the amount of secreted IL-8 and IL-6 protein. The amount of IL-8 protein produced from the low and high PL cultures following LPS exposure (863 ± 184 vs. 969 ± 241 pg/ml, respectively; Figure 1A) and IL-1β treatment (5664 ± 685 vs. 6161 ± 1036 pg/ml, respectively; Figure 1B) was not significantly different. Similarly, levels

of secreted IL-6 protein following LPS or IL-1β challenges were not different between low and high PL cultures (Figure 2A and 2B).

A.4 Discussion

In this study, dermal fibroblasts were isolated from animals with different productive life (PL) values and challenged with LPS to determine if we could predict an animal's innate immune response phenotype based on the PL values assigned to her. This trait is an indicator of how many additional months an animal may remain in the herd compared to her herd-mates. Since longevity in a dairy herd requires a combination of health and conformation traits that minimize disease risk while maximizing milk production, an animal with a greater PL may have a lower propensity to succumb to a severe mammary infection that could result in removal from the herd. Surprisingly, fibroblast cultures established from the two groups of animals (low and high PL) had similar IL-8 and IL-6 protein responses following the LPS challenge, suggesting that PL may not be the best trait to predict the innate immune response phenotype of an animal.

The most common trait that producers utilize to select animals for greater mastitis resistance is somatic cell count (SCC). This involves collecting a composite milk sample once a month to determine the level of immune cells present in the mammary gland. Within a healthy gland there are some resident immune cells readily available to sense and respond to an invading pathogen, and the benchmark SCC for a healthy gland is 200,000 cells/ml of milk (or lower). However, during a case of mastitis, the SCC will increase dramatically in the infected quarter due to the recruitment of neutrophils to clear the infection (Wellnitz and Bruckmaier, 2012), and in some cases this rise in SCC can be

detected by the monthly SCC testing. However, the increase in SCC due to some mammary infections, especially coliforms like *E. coli*, can be missed as the infection can be cleared in a matter of days (Bannerman et al., 2008, Kandasamy et al., 2011). Even if the infected quarter still has an elevated SCC at the time of sample collection, the composite SCC could be close to a healthy SCC, which may result in that animal being inadvertently classified as an un-infected animal that has higher mastitis resistance than her herd-mates. Including other traits, such as mastitis severity, may increase the accuracy of selecting for animals that mount an effective yet more controlled inflammatory response towards mastitis.

In conclusion, the similar in vitro response between the low and high PL fibroblast cultures suggests that PL may not be an accurate predictor of the innate immune response phenotype of an animal. However, the animals on the collaborating dairy farm have undergone genomic testing and selection for several generations, creating a herd of cattle with higher genetic merit than the average commercial dairy herd. Therefore, even animals with a low PL (for this herd) may still have a higher PL compared to the average commercial Holstein cow. Additionally, the lower numbers of animals in each group may have limited our ability to detect differences in the in vitro innate immune response. Due to the high worth of these cattle, we were limited in the number of animals available for fibroblast collection and in vivo challenges were not an option.

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Table 1 Productive life (PL) values and birth dates for the selected animals.

Animal ID	Birth Date	Productive Life (PL)
Low Productive Life Animals		
2706	7/11/08	-0.7
2510	8/26/07	-0.3
2699	7/2/08	0.3
2705	7/11/08	1.2
2590	12/17/07	1.3
High Productive Life Animals		
2683	6/10/08	5.1
2610	1/25/08	5.1
2793	10/23/08	5.2
2563	11/16/07	5.3
2629	3/18/08	5.6
2472	7/7/07	6.3

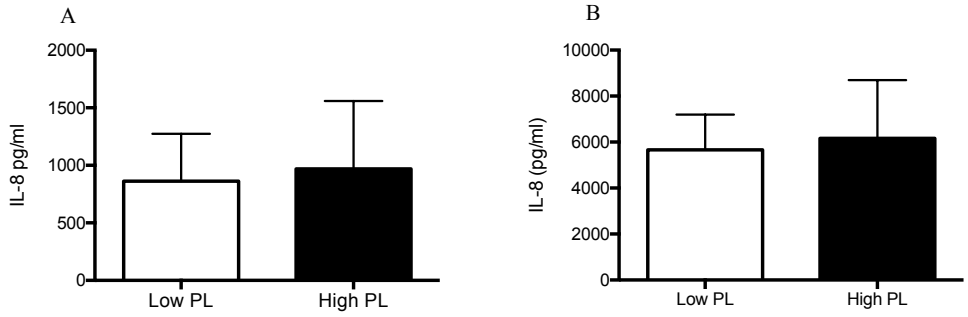

Figure 1 IL-8 protein production from dermal fibroblasts following LPS and IL-1β challenges.

Aliquots of fibroblasts isolated from animals with a high productive life (PL) value (n=6) and animals with a low PL (n=5) were revived and challenged in parallel for 24 h with LPS (100ng/ml; A) or IL-1β (1ng/ml; B). Media was collected and the amount of secreted IL-8 protein was quantified via ELISA. Values are mean ± SEM. There were no significant differences between low and high PL cultures in the amount of IL-8 produced.

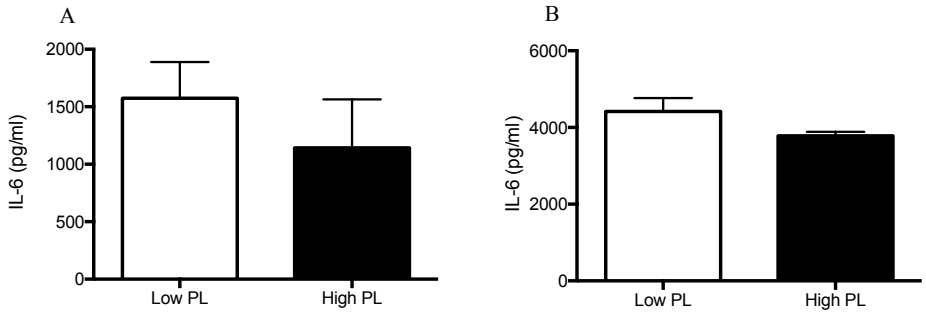

Figure 2 IL-6 protein production from dermal fibroblasts following LPS and IL-1β challenges.

Aliquots of fibroblasts isolated from animals with a high productive life (PL) value (n=6) and animals with a low PL (n=5) were revived and challenged in parallel for 24 h with LPS (100ng/ml; A) or IL-1β (1ng/ml; B). Media was collected and the amount of secreted IL-6 protein was quantified via ELISA. Values are mean ± SEM. There were no significant differences between low and high PL cultures in the amount of IL-6 produced.

Appendix B: A comparison of LPS-response of dermal fibroblasts isolated from young Angus and Holstein animals

B.1 Experimental Design and Rationale

Within our dermal fibroblast cellular model we observed a range in the IL-8 protein responses following LPS challenge. Based on the wide range in the amount of IL-8 produced, we have classified the extremes of this range as low and high responding phenotypes, and went on to confirm that these in vitro phenotypic classifications are maintained in vivo during an intra-mammary challenge (Kandasamy et al., 2011). Similarly, Green and Kerr (2014) observed low and high IL-8 response phenotypes from fibroblast cultures established from the same animal at 5 and 16 months of age. Cultures from the young animals had a much lower IL-8 response following an LPS challenge compared to cultures from the older animals. Expanding on the phenotypic differences, we observed that fibroblasts from 19 month old Angus animals exhibited a lower IL-8 protein response following an LPS challenge compared to fibroblasts from age-matched Holstein animals (Benjamin et al., 2016). The aim of this study was to confirm the breed-difference observed previously in a second set of fibroblast cultures established from younger animals.

B.2 Materials and Methods

B.2.1 Dermal Fibroblast Isolation and Challenge

Skin biopsies were collected from Angus (n=7; age 13.2 ± 0.46 months) and Holstein (n=8; age 15.2 ± 0.26 months) animals and dermal fibroblasts were isolated as previously described (Kandasamy et al., 2011). Following a 6 h digestion with collagenase type I (Life Technologies, Grand Island, NY), fibroblasts were seeded into 25-cm^2 flask (Corning Inc., Corning, NY) in Dulbecco's Modified Eagle Medium

(DMEM; Hyclone Laboratories, Logan, UT) with 10% fetal bovine serum (FBS; Hyclone Laboratories), 1X antibiotic cocktail (100 U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin; Hyclone Laboratories), and 1X Insulin-Transferrin-Selenium (ITS; Mediatech Inc., Herndon, VA). Upon confluency, cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded into a 75-cm^2 flask (Corning Inc.) in DMEM with 5% FBS, 1X antibiotic cocktail, and 1X ITS. After approximately three days, cells were expanded into three 75-cm^2 flasks, and once confluent, cells were detached from the flasks with trypsin, diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma- Aldrich, St. Louis, MO), and cryopreserved in liquid nitrogen for subsequent challenges.

Aliquots of fibroblasts were revived from liquid nitrogen, seeded into a 75-cm^2 flask, and incubated at 37°C with 5% CO_2 for three days to allow for adequate cell expansion. Cells were detached with trypsin, counted, and seeded into 6-well plates (Corning Inc.) at 1.25 x 10^5 cells/ml in a total volume of 2ml. Media was replaced 24 h later with 2 ml of either fresh media, media containing 100ng/ml of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich), or media containing 1ng/ml of recombinant bovine IL-1β (AbD Serotech, Raleigh, NC). Following a 36 h challenge, media was collected from each well, centrifuged at 10,000 x *g* for 1 min to remove cellular debris and stored at -20°C for IL-8 protein analysis.

B.2.2 IL-8 Quantification

The concentration of IL-8 in conditioned media samples was determined by a custom sandwich ELISA as described previously (Kandasamy and Kerr, 2012). Mouse

anti-bovine (clone 170.13, gifted by Samuel Maheswaren, University of Minnesota, St. Paul, MN) and a biotinylated goat anti-human IL-8 (R&D Systems Inc., Minneapolis, MN) were used as capture and detection antibodies, respectively. Recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) was used as the assay standard. The detection limit for this assay was 130pg/ml.

B.2.3 Statistical Analysis

Differences in IL-8 protein production between Angus and Holstein fibroblast cultures were determined using a Student's t-test (Graph Pad Prism 6.0).

B.3 Results

Fibroblasts isolated from young Angus (13.2 m) and Holstein (15.2 m) animals were challenged for 36 h with either LPS (100ng/ml) or IL-1β (1ng/ml) and IL-8 protein was quantified. Angus cultures produced significantly lower levels of IL-8 following LPS exposure compared to Holstein cultures (74 ± 38 vs. 453 ± 78 pg/ml, respectively; Figure 1). A similar response was observed following IL-1β treatment, with Angus cultures producing less IL-8 than Holstein cultures (Figure 1).

B.4 Discussion

In this study, dermal fibroblasts were isolated from young Angus (13.2 m) and Holstein (15.2 m) animals to confirm breed-differences in the fibroblast LPS response observed previously (Benjamin et al., 2016). Similar to the low IL-8 response phenotype previously observed in cultures from 19 month old Angus animals, cultures isolated from

13 m old Angus produced significantly less IL-8 protein post-LPS exposure compared to 15 month-old Holsteins. A potential drawback of this experiment was the two-month, significant ($P < 0.05$) difference in age between the breed groups. Given that we have previously reported an age-dependent increase in fibroblast responsiveness (Green et al., 2011), a portion of the observed breed difference could have been due to an age difference. However, we are confident that the breed-difference observed in 19 month old animals was confirmed in a set of fibroblast cultures isolated from younger Angus and Holstein animals.

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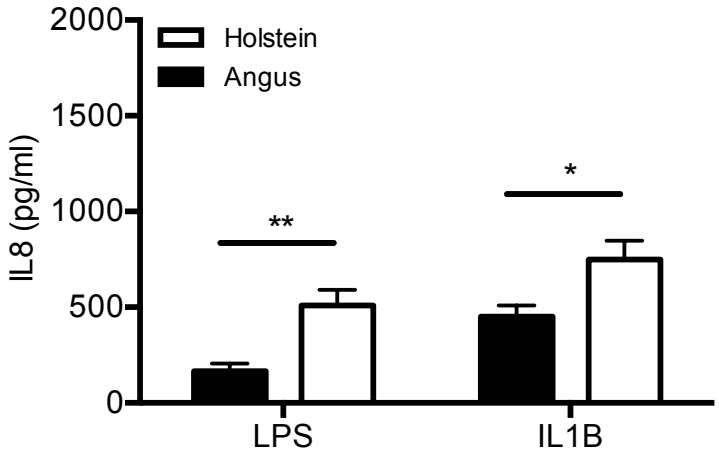

Figure 1 IL-8 protein production from Angus and Holstein dermal fibroblasts.

Dermal fibroblasts were isolated from Angus (n=7; age 13.2 m) and Holstein (n=8; age 15.2 m) animals and challenged for 36 h with either LPS (100ng/ml) or IL-1β (1ng/ml). Media was collected post-challenge from each well and the amount of IL-8 protein was determined via ELISA. Wells containing plain media (negative control) had no detectable IL-8. Values are mean ± SEM and ** indicates a $P < 0.01$.

Appendix C: Detailed methods for IV catheterization prior to an *in vivo* LPS challenge

C.1 Methodology Rationale

This appendix contains a detailed description of the methodology for inserting an intravenous (IV) catheter in the jugular vein of an animal to facilitate blood sampling and administration of lipopolysaccharide (LPS) during a challenge.

C.2 Catheterization Method

Twelve hours prior to an IV LPS challenge, each animal was fitted with a jugular catheter to allow for easier administration of the LPS solution and blood sampling. Twenty-four hours prior to the catheterization, a 50 cm section of polyethylene tubing (I.D. 1.19mm, O.D. 1.70mm; Intramedic, BD) was cut per animal and sanitized overnight in a Nolvasan S solution (Pfizer, NY, NY). The day of catheterization each animal was secured in a headlock and a small square patch of fur on top of the jugular vein was clipped and the area was cleansed with 70% ethanol. The polyethylene tubing was prepared by rinsing and flushing out the Nolvasan solution with sterile saline. A 12-gauge, 1.5-inch metal hub needle (Hamilton, Reno, NV) was then inserted into the jugular vein, and upon visualization of blood through the needle, 20-25 cm of the polyethylene tubing was inserted through the needle. The needle was carefully removed from the skin, leaving the tubing in the jugular vein. A 7.6 x 7.6 cm square piece of veterinary elastic adhesive tape (3M, St. Paul, MN) was prepared by cutting a small hole in the center (for the tubing), and a small amount of Kamar adhesive (Kamar Products, Inc., Zionsville, IN) was applied to the non-adhesive side of the elastic tape. The needle was pulled along the length of the tubing and removed, and then a small 2.5 x 2.5 cm square of white medical tape was folded over the tubing at the entry point to act as a reference point for the length

of tubing in the jugular vein. The tubing was then carefully fed through the small opening in the tape and the Kamar adhesive was stuck to the animal's neck over the site where the tube entered the skin. An 18-guage luer stub adapter (Intramedic) was attached to the free end of the tubing and a sterile syringe was used to draw a small amount of blood to ensure the tubing was still properly placed. Once this was confirmed, a solution of heparinized saline was injected to prevent clotting in the tubing. A three-way stopcock with Swivel Male Luer Lock (Smiths Medical ASD, Inc., Dublin, OH) was then attached to the adapter to allow for infusion of the LPS solution as well as drawing of samples. Finally, the excess tubing was loosely coiled and two rolls of vet wrap (SyrVet, Waukee, IA) were wrapped around the animal's neck to protect the catheter site and contain the excess tubing. A single strip of white adhesive medical tape was wrapped loosely around the animal's neck to secure the vet wrap. The animal was rested for ~12 hours. The following morning, each catheter was inspected to ensure it was functional prior to the start of the LPS challenge.